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(54) Title: CHEMOKINE RECEPTORS 88-2B[CKR-3] AND 88C AND THEIR ANTIBODIES

(57) Abstract

The present invention provides polynucleotides that encode the chemokine receptors 88-2B or 88C and materials and methods for the recombinant production of these two chemokine receptors. Also provided are assays utilizing the polynucleotides which facilitate the identification of ligands and modulators of the chemokine receptors. Receptor fragments, ligands, modulators, and antibodies are useful in the detection and treatment of disease states associated with the chemokine receptors such as atherosclerosis, rheumatoid arthritis, tumor growth suppression, asthma, viral infection, AIDS, and other inflammatory conditions.

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**CHEMOKINE RECEPTORS 88-2B [CKR-3] AND 88C AND THEIR ANTIBODIES**

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/661,393 filed June 7, 1996 which was in turn a 5 continuation-in-part of U.S. Patent Application No. 08/575,967 filed December 20, 1995.

**FIELD OF THE INVENTION**

The present invention relates generally to signal transduction pathways. More particularly, the present invention relates to chemokine receptors, nucleic acids encoding chemokine receptors, chemokine receptor ligands, modulators of chemokine receptor activity, antibodies recognizing chemokines and chemokine receptors, methods for identifying chemokine receptor ligands and modulators, methods for producing chemokine receptors, and methods for producing antibodies recognizing chemokine receptors.

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**BACKGROUND OF THE INVENTION**

Recent advances in molecular biology have led to an appreciation of the central role of signal transduction pathways in biological processes. These pathways comprise a central means by which individual cells in a multicellular organism communicate, thereby coordinating biological processes. See *Springer, Cell* 76:301-314 (1994), Table I for a model. One branch of signal transduction pathways, defined by the intracellular participation of guanine nucleotide binding proteins (G-proteins), affects a broad range of biological processes.

20 *Lewin, GENES* V 319-348 (1994) generally discusses G-protein signal transduction pathways which involve, at a minimum, the following components: an extracellular signal (e.g., neurotransmitters, peptide hormones, organic molecules, light, or odorants), a signal-recognizing receptor (G-protein-coupled receptor, reviewed in *Probst et al., DNA and Cell Biology* 11:1-20 [1992] and also known as GPR or GPCR), and an intracellular heterotrimeric GTP-binding protein, or G protein. In particular, these

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pathways have attracted interest because of their role in regulating white blood cell or leukocyte trafficking.

Leukocytes comprise a group of mobile blood cell types including granulocytes (*i.e.*, neutrophils, basophils, and eosinophils), lymphocytes, and monocytes. When mobilized and activated, these cells are primarily involved in the body's defense against foreign matter. This task is complicated by the diversity of normal and pathological processes in which leukocytes participate. For example, leukocytes function in the normal inflammatory response to infection. Leukocytes are also involved in a variety of pathological inflammations. For a summary, see *Schall et al., Curr. Opin. Immunol.* 6:865-873 (1994). Moreover, each of these processes can involve unique contributions, in degree, kind, and duration, from each of the leukocyte cell types.

In studying these immune reactions, researchers initially concentrated on the signals acting upon leukocytes, reasoning that a signal would be required to elicit any form of response. *Murphy, Ann. Rev. Immunol.* 12:593-633 (1994) has reviewed members of an important group of leukocyte signals, the peptide signals. One type of peptide signal comprises the chemokines (*chemoattractant cytokines*), termed intercrines in *Oppenheim et al., Ann. Rev. Immunol.* 9:617-648 (1991). In addition to *Oppenheim et al., Baggolini et al., Advances in Immunol.* 55:97-179 (1994), documents the growing number of chemokines that have been identified and subjected to genetic and biochemical analyses.

Comparisons of the amino acid sequences of the known chemokines have led to a classification scheme which divides chemokines into two groups: the  $\alpha$  group characterized by a single amino acid separating the first two cysteines (CXC; N-terminus as referent), and the  $\beta$  group, where these cysteines are adjacent (CC). See *Baggolini et al., supra*. Correlations have been found between the chemokines and the particular leukocyte cell types responding to those signals. *Schall et al., supra*, has reported that the CXC chemokines generally affect neutrophils; the CC chemokines tend to affect monocytes.

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lymphocytes, basophils and eosinophils. For example, *Baggiolini et al.*, *supra*, recited that RANTES, a CC chemokine, functions as a chemoattractant for monocytes, lymphocytes (*i.e.*, memory T cells), basophils, and eosinophils, but not for neutrophils, while inducing the release of histamine from basophils.

Chemokines were recently shown by Cocchi, *et. al.*, *Science*, 270: 1811-1815 (1995) to be suppressors of HIV proliferation. Cocchi, *et al.* demonstrated that RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  suppressed HIV-1, HIV-2 and SIV infection of a CD4 $^{+}$  cell line designated PM1 and of primary human peripheral blood mononuclear cells.

Recently, however, attention has turned to the cellular receptors that bind the chemokines, because the extracellular chemokines seem to contact cells indiscriminately, and therefore lack the specificity needed to regulate the individual leukocyte cell types.

*Murphy, supra*, reported that the GPCR superfamily of receptors includes the chemokine receptor family. The typical chemokine receptor structure includes an extracellular chemokine-binding domain located near the N-terminus, followed by seven spaced regions of predominantly hydrophobic amino acids capable of forming membrane-spanning  $\alpha$ -helices. Between each of the  $\alpha$ -helical domains are hydrophilic domains localized, alternately, in the intra- or extra-cellular spaces. These features impart a serpentine conformation to the membrane-embedded chemokine receptor. The third intracellular loop typically interacts with G-proteins. In addition, *Murphy, supra*, noted that the intracellular carboxyl terminus is also capable of interacting with G-proteins.

The first chemokine receptors to be analyzed by molecular cloning techniques were the two neutrophil receptors for human IL8, a CXC chemokine. *Holmes et al.*, *Science* 253:1280 (1991) and *Murphy et al.*, *Science* 253:1280-1283 (1991), reported the cloning of these two receptors for IL8. *Lee et al.*, *J. Biol. Chem.* 267:16283-16287 (1992), analyzed the cDNAs encoding these receptors and found 77% amino acid identity between the encoded receptors, with each receptor exhibiting features of the G protein

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coupled receptor family. One of these receptors is specific for IL-8, while the other binds and signals in response to IL-8, gro/MGSA, and NAP-2. Genetic manipulation of the genes encoding IL-8 receptors has contributed to our understanding of the biological roles occupied by these receptors. For example, *Cacalano et al.*, *Science* 265:682-684 (1994) reported that deletion of the IL-8 receptor homolog in the mouse resulted in a pleiotropic phenotype involving lymphadenopathy and splenomegaly. In addition, a study of missense mutations described in *Leong et al.*, *J. Biol. Chem.* 269:19343-19348 (1994) revealed amino acids in the IL-8 receptor that were critical for IL-8 binding. Domain swapping experiments discussed in *Murphy, supra*, implicated the amino terminal extracellular domain as a determinant of binding specificity.

Several receptors for CC chemokines have also been identified and cloned. CCCKR1 binds both MIP-1 $\alpha$  and RANTES and causes intracellular calcium ion flux in response to both ligands. Charo *et al.*, *Proc Natl. Acad. Sci. (USA)* 91:2752-2756 (1994) reported that another CC chemokine receptor, MCP-R1 (CCCKR2), is encoded by a single gene that produces two splice variants which differ in their carboxyl terminal domains. This receptor binds and responds to MCP-3 in addition to MCP-1.

20 A promiscuous receptor that binds both CXC and CC chemokines has also been identified. This receptor was originally identified on red blood cells and Horuk *et al.*, *Science* 261:1182-1184 (1993) reports that it binds IL-8, NAP-2, GRO $\alpha$ , RANTES, and MCP-1. The erythrocyte chemokine receptor shares about 25% identity with other chemokine receptors  
25 and may help to regulate circulating levels of chemokines or aid in the presentation of chemokines to their targets. In addition to binding chemokines, the erythrocyte chemokine receptor has also been shown to be the receptor for plasmodium vivax, a major cause of malaria (id.). Another G-protein coupled receptor which is closely related to chemokine receptors, the  
30 platelet activating factor receptor, has also been shown to be the receptor for a human pathogen, the bacterium *Streptococcus pneumoniae* (Cundell *et al.*, *Nature* 377:435-438 (1995)).

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In addition to the mammalian chemokine receptors, two viral chemokine receptor homologs have been identified. Ahuja *et al.*, *J. Biol. Chem.* 268:20691-20694 (1993) describes a gene product from *Herpesvirus saimiri* that shares about 30% identity with the IL-8 receptors and binds CXC chemokines. Neote *et al.*, *Cell*, 72:415-425 (1993) reports that human cytomegalovirus contains a gene encoding a receptor sharing about 30% identity with the CC chemokine receptors which binds MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES. These viral receptors may affect the normal role of chemokines and provide a selective pathological advantage for the virus.

Because of the broad diversity of chemokines and their activities, there are numerous receptors for the chemokines. The receptors which have been characterized represent only a fraction of the total complement of chemokine receptors. There thus remains a need in the art for the identification of additional chemokine receptors. The availability of these novel receptors will provide tools for the development of therapeutic modulators of chemokine or chemokine receptor function. It is contemplated by the present invention that such modulators are useful as therapeutics for the treatment of atherosclerosis, rheumatoid arthritis, tumor growth suppression, asthma, viral infections, and other inflammatory conditions. Alternatively, fragments or variants of the chemokine receptors, or antibodies recognizing those receptors, are contemplated as therapeutics.

### SUMMARY OF THE INVENTION

The present invention provides purified and isolated nucleic acids encoding chemokine receptors involved in leukocyte trafficking. Polynucleotides of the invention (both sense and anti-sense strands thereof) include genomic DNAs, cDNAs, and RNAs, as well as completely or partially synthetic nucleic acids. Preferred polynucleotides of the invention include the DNA encoding the chemokine receptor 88-2B that is set out in SEQ ID NO:3, the DNA encoding the chemokine receptor 88C that is set out in SEQ ID NO:1, and DNAs which hybridize to those DNAs under standard stringent hybridization conditions, or which would hybridize but for the redundancy of

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the genetic code. Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM sodium phosphate, pH 6.8 and washing in 0.2X SSC at 55°C. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See *Sambrook et al.*, §§ 9.47-9.51 in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). Also contemplated by the invention are polynucleotides encoding domains of 88-2B or 88C, for example, polynucleotides encoding one or more extracellular domains of either protein or other biologically active fragments thereof. 88-2B extracellular domains correspond to SEQ ID NO:3 and SEQ ID NO:4 at amino acid residues 1-36, 93-107, 171-196, and 263-284. The extracellular domains of 88-2B are encoded by polynucleotide sequences corresponding to SEQ ID NO:3 at nucleotides 362-469, 638-682, 872-949, and 1148-1213. Extracellular domains of 88C correspond to SEQ ID NO:1 and SEQ ID NO:2 at amino acid residues 1-32, 89-112, 166-191, and 259-280. The 88C extracellular domains are encoded by polynucleotide sequences that correspond to SEQ ID NO:1 at nucleotides 55-150, 319-390, 550-627, and 829-894. The invention also comprehends polynucleotides encoding intracellular domains of these chemokine receptors. The intracellular domains of 88-2B include amino acids 60-71, 131-151, 219-240, and 306-355 of SEQ ID NO:3 and SEQ ID NO:4. Those domains are encoded by polynucleotide sequences corresponding to SEQ ID NO:3 at nucleotides 539-574, 752-814, 1016-1081, and 1277-1426, respectively. The 88C intracellular domains include amino acid residues 56-67, 125-145, 213-235, and 301-352 of SEQ ID NO:1 and SEQ ID NO:2. The intracellular domains of 88C are encoded by polynucleotide sequences corresponding to SEQ ID NO:1 at nucleotides 220-255, 427-489, 691-759, and 955-1110. Peptides corresponding to one or more of the extracellular or intracellular domains, or antibodies raised against those peptides, are contemplated as modulators of receptor activities, especially ligand and G protein binding activities of the receptors.

The nucleotide sequences of the invention may also be used to design oligonucleotides for use as labeled probes to isolate genomic DNAs encoding 88-2B or 88C under stringent hybridization conditions (*i.e.*, by Southern analyses and Polymerase Chain Reaction methodologies). Moreover, 5 these oligonucleotide probes can be used to detect particular alleles of the genes encoding 88-2B or 88C, facilitating both diagnosis and gene therapy treatments of disease states associated with particular alleles. In addition, these oligonucleotides can be used to alter chemokine receptor genetics to facilitate identification of chemokine receptor modulators. Also, the 10 nucleotide sequences can be used to design antisense genetic elements of use in exploring or altering the genetics and expression of 88-2B or 88C. The invention also comprehends biological replicas (*i.e.*, copies of isolated DNAs made *in vivo* or *in vitro*) and RNA transcripts of DNAs of the invention. Autonomously replicating recombinant constructions such as plasmid, viral, 15 and chromosomal (*e.g.*, YAC) nucleic acid vectors effectively incorporating 88-2B or 88C polynucleotides, and, particularly, vectors wherein DNA effectively encoding 88-2B or 88C is operatively linked to one or more endogenous or heterologous expression control sequences are also provided.

The 88-2B and 88C receptors may be produced naturally, 20 recombinantly or synthetically. Host cells (prokaryotic or eukaryotic) transformed or transfected with polynucleotides of the invention by standard methods may be used to express the 88-2B and 88C chemokine receptors. Beyond the intact 88-2B or 88C gene products, biologically active fragments 25 of 88-2B or 88C, analogs of 88-2B or 88C, and synthetic peptides derived from the amino acid sequences of 88-2B, set out in SEQ ID NO:4, or 88C, set out in SEQ ID NO:2, are contemplated by the invention. Moreover, the 88-2B or 88C gene product, or a biologically active fragment of either gene product, when produced in a eukaryotic cell, may be post-translationally modified (*e.g.*, via disulfide bond formation, glycosylation, phosphorylation, myristoylation, palmitoylation, acetylation, etc.) The invention further 30 contemplates the 88-2B and 88C gene products, or biologically active

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fragments thereof, in monomeric, homomultimeric, or heteromultimeric conformations.

In particular, one aspect of the invention involves antibody products capable of specifically binding to the 88-2B or 88C chemokine receptors. The antibody products are generated by methods standard in the art using recombinant 88-2B or 88C receptors, synthetic peptides or peptide fragments of 88-2B or 88C receptors, host cells expressing 88-2B or 88C on their surfaces, or 88-2B or 88C receptors purified from natural sources as immunogens. The antibody products may include monoclonal antibodies or polyclonal antibodies of any source or sub-type. Moreover, monomeric, homomultimeric, and heteromultimeric antibodies, and fragments thereof, are contemplated by the invention. Further, the invention comprehends CDR-grafted antibodies, "humanized" antibodies, and other modified antibody products retaining the ability to specifically bind a chemokine receptor.

The invention also contemplates the use of antibody products for detection of the 88-2B or 88C gene products, their analogs, or biologically active fragments thereof. For example, antibody products may be used in diagnostic procedures designed to reveal correlations between the expression of 88-2B, or 88C, and various normal or pathological states. In addition, antibody products can be used to diagnose tissue-specific variations in expression of 88-2B or 88C, their analogs, or biologically active fragments thereof. Antibody products specific for the 88-2B and 88C chemokine receptors may also act as modulators of receptor activities. In another aspect, antibodies to 88-2B or 88C receptors are useful for therapeutic purposes.

Assays for ligands capable of interacting with the chemokine receptors of the invention are also provided. These assays may involve direct detection of chemokine receptor activity, for example, by monitoring the binding of a labeled ligand to the receptor. In addition, these assays may be used to indirectly assess ligand interaction with the chemokine receptor. As used herein the term "ligand" comprises molecules which are agonists and antagonists of 88-2B or 88C, and other molecules which bind to the receptors.

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Direct detection of ligand binding to a chemokine receptor may be achieved using the following assay. Test compounds (*i.e.*, putative ligands) are detectably labeled (*e.g.*, radioiodinated). The detectably labeled test compounds are then contacted with membrane preparations containing a chemokine receptor of the invention. Preferably, the membranes are prepared from host cells expressing chemokine receptors of the invention from recombinant vectors. Following an incubation period to facilitate contact between the membrane-embedded chemokine receptors and the detectably labeled test compounds, the membrane material is collected on filters using vacuum filtration. The detectable label associated with the filters is then quantitated. For example, radiolabels are quantitated using liquid scintillation spectrophotometry. Using this technique, ligands binding to chemokine receptors are identified. To confirm the identification of a ligand, a detectably labeled test compound is exposed to a membrane preparation displaying a chemokine receptor in the presence of increasing quantities of the test compound in an unlabeled state. A progressive reduction in the level of filter-associated label as one adds increasing quantities of unlabeled test compound confirms the identification of that ligand.

Agonists are ligands which bind to the receptor and elicit intracellular signal transduction and antagonists are ligands which bind to the receptor but do not elicit intracellular signal transduction. The determination of whether a particular ligand is an agonist or an antagonist can be determined, for example, by assaying G protein-coupled signal transduction pathways. Activation of these pathways can be determined by measuring intracellular  $\text{Ca}^{++}$  flux, phospholipase C activity or adenylyl cyclase activity, in addition to other assays (see examples 5 and 6).

As discussed in detail in the Examples herein, chemokines that bind to the 88C receptor include RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ , and chemokines that bind to the 88-2B receptor include RANTES.

In another aspect, modulators of the interaction between the 88C and 88-2B receptors and their ligands are specifically contemplated by the invention. Modulators of chemokine receptor function may be identified using

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assays similar to those used for identifying ligands. The membrane preparation displaying a chemokine receptor is exposed to a constant and known quantity of a detectably labeled functional ligand. In addition, the membrane-bound chemokine receptor is also exposed to an increasing quantity of a test compound suspected of modulating the activity of that chemokine receptor. If the levels of filter-associated label correlate with the quantity of test compound, that compound is a modulator of the activity of the chemokine receptor. If the level of filter-associated label increases with increasing quantities of the test compound, an activator has been identified. In contrast, if the level of filter-associated label varies inversely with the quantity of test compound, an inhibitor of chemokine receptor activity has been identified. Testing for modulators of receptor binding in this way allows for the rapid screening of many putative modulators, as pools containing many potential modulators can be tested simultaneously in the same reaction.

The indirect assays for receptor binding involve measurements of the concentration or level of activity of any of the components found in the relevant signal transduction pathway. Chemokine receptor activation often is associated with an intracellular  $\text{Ca}^{++}$  flux. Cells expressing chemokine receptors may be loaded with a calcium-sensitive dye. Upon activation of the expressed receptor, a  $\text{Ca}^{++}$  flux would be rendered spectrophotometrically detectable by the dye. Alternatively, the  $\text{Ca}^{++}$  flux could be detected microscopically. Parallel assays, using either technique, may be performed in the presence and absence of putative ligands. For example, using the microscopic assay for  $\text{Ca}^{++}$  flux, RANTES, a CC chemokine, was identified as a ligand of the 88-2B chemokine receptor. Those skilled in the art will recognize that these assays are also useful for identifying and monitoring the purification of modulators of receptor activity. Receptor activators and inhibitors will activate or inhibit, respectively, the interaction of the receptors with their ligands in these assays.

Alternatively, the association of chemokine receptors with G proteins affords the opportunity of assessing receptor activity by monitoring

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G protein activities. A characteristic activity of G proteins, GTP hydrolysis, may be monitored using, for example, <sup>32</sup>P-labeled GTP.

G proteins also affect a variety of other molecules through their participation in signal transduction pathways. For example, G protein effector molecules include adenylyl cyclase, phospholipase C, ion channels, and phosphodiesterases. Assays focused on any of these effectors may be used to monitor chemokine receptor activity induced by ligand binding in a host cell that is both expressing the chemokine receptor of interest and contacted with an appropriate ligand. For example, one method by which the activity of chemokine receptors may be detected involves measuring phospholipase C activity. In this assay, the production of radiolabeled inositol phosphates by host cells expressing a chemokine receptor in the presence of an agonist is detected. The detection of phospholipase activity may require cotransfection with DNA encoding an exogenous G protein. When cotransfection is required, this assay can be performed by cotransfection of chimeric G protein DNA, for example, Gqi5 (*Conklin, et al., Nature* 363:274-276 (1993)), with 88-2B or 88C DNA and detecting phosphoinositol production when the cotransfected cell is exposed to an agonist of the 88-2B or 88C receptor. Those skilled in the art will recognize that assays focused on G-protein effector molecules are also useful for identifying and monitoring the purification of modulators of receptor activity. Receptor activators and inhibitors will activate or inhibit, respectively, the interaction of the receptors with their ligands in these assays.

Chemokines have been linked to many inflammatory diseases, such as psoriasis, arthritis, pulmonary fibrosis and atherosclerosis. See *Baggiolini et al., supra*. Inhibitors of chemokine action may be useful in treating these conditions. In one example, *Broaddus et al., J. of Immunol.* 152:2960-2967 (1994), describes an antibody to IL-8 which can inhibit neutrophil recruitment in endotoxin-induced pleurisy, a model of acute inflammation in rabbit lung. It is also contemplated that ligand or modulator binding to, or the activation of, the 88C receptor may be useful in treatment of HIV infection and HIV related disease states. Modulators of chemokine

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binding to specific receptors contemplated by the invention may include antibodies directed toward a chemokine or a receptor, biological or chemical small molecules, or synthetic peptides corresponding to fragments of the chemokine or receptor.

5        Administration of compositions containing 88-2B or 88C modulators to mammalian subjects, for the purpose of monitoring or remediating normal or pathological immune reactions And viral infections including infection by retroviruses such as HIV-1, HIV-2 and SIV is contemplated by the invention. In particular, the invention comprehends the  
10 mitigation of inflammatory responses, abnormal hematopoietic processes, and viral infections by delivery of a pharmaceutically acceptable quantity of 88-2B or 88C chemokine receptor modulators. The invention further comprehends delivery of these active substances in pharmaceutically acceptable compositions comprising carriers, diluents, or medicaments. The invention  
15 also contemplates a variety of administration routes. For example, the active substances may be administered by the following routes: intravenous, subcutaneous, intraperitoneal, intramuscular, oral, anal (*i.e.*, via suppository formulations), or pulmonary (*i.e.*, via inhalers, atomizers, nebulizers, etc.)

In another aspect, the DNA sequence information provided by  
20 the present invention makes possible the development, by homologous recombination or "knockout" strategies [see, e.g. Kapecchi, *Science*, 244:1288-1292 (1989)], of rodents that fail to express a functional 88C or 88-2B chemokine receptor or that express a variant of the receptor. Alternatively, transgenic mice which express a cloned 88-2B or 88C receptor can be prepared by well known laboratory techniques (Manipulating the  
25 Mouse Embryo: A Laboratory Manual, Brigid Hohan, Frank Costantini and Elizabeth Lacy, eds. (1986) Cold Spring Harbor Laboratory ISBN 0-87969-175-1). Such rodents are useful as models for studying the activities of 88C or 88-2B receptors *in vivo*.

30        Other aspects and advantages of the present invention will become apparent to one skilled in the art upon consideration of the following examples.

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### DETAILED DESCRIPTION OF THE INVENTION

The following examples illustrate the invention. Example 1 describes the isolation of genomic DNAs encoding the 88-2B and 88C chemokine receptors. Example 2 presents the isolation and sequencing of cDNAs encoding human 88-2B and 88C and macaque 88C. Example 3 provides a description of Northern analyses revealing the expression patterns of the 88-2B and 88C receptors in a variety of tissues. Example 4 details the recombinant expression of the 88-2B and 88C receptors. Example 5 describes Ca<sup>++</sup> flux assays, phosphoinositol hydrolysis assays, and binding assays for 88-2B and 88C receptor activity in response to a variety of potential ligands. Experiments describing the role of 88C and 882B as co-receptors for HIV is presented in Examples 6 and 7. The preparation and characterization of monoclonal and polyclonal antibodies immunoreactive with 88C is described in Example 8. Example 9 describes additional assays designed to identify 88-2B or 88C ligands or modulators.

#### Example 1

Partial genomic clones encoding the novel chemokine receptor genes of this invention were isolated by PCR based on conserved sequences found in previously identified genes and based on a clustering of these chemokine receptor genes within the human genome. The genomic DNA was amplified by standard PCR methods using degenerate oligonucleotide primers.

Templates for PCR amplifications were members of a commercially available source of recombinant human genomic DNA cloned into Yeast Artificial Chromosomes (*i.e.*, YACs). (Research Genetics, Inc., Huntsville, AL. YAC Library Pools, catalog no. 95011 B). A YAC vector can accommodate inserts of 500-1000 kilobase pairs. Initially, pools of YAC clone DNAs were screened by PCR using primers specific for the gene encoding CCCKR1. In particular, CCCKR(2)-5', the sense strand primer (corresponding to the sense strand of CCCKR1), is presented in SEQ ID NO:15. Primer CCCKR(2)-5' consisted of the sequence 5'-CGTAAGCTTAGAGAAGCCGGGATGGGAA-3', wherein the underlined

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nucleotides are the translation start codon for CCCKR1. The anti-sense strand primer was CCCKR-3' (corresponding to the anti-sense strand of CCCKR1) and its sequence is presented in SEQ ID NO:16. The sequence of CCCKR-3', 5'-GCCTCTAGAGTCAGAGACCAGCAGA-3', contains the reverse complement of the CCCKR1 translation stop codon (underlined). Pools of YAC clone DNAs yielding detectable PCR products (*i.e.*, DNA bands upon gel electrophoresis) identified appropriate sub-pools of YAC clones, based on a proprietary identification scheme. (Research Genetics, Inc., Huntsville, AL). PCR reactions were initiated with an incubation at 94°C for four minutes. Sequence amplifications were achieved using 33 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for two minutes.

The sub-pools of YAC clone DNAs were then subjected to a second round of PCR reactions using the conditions, and primers, that were used in the first round of PCR. Results from sub-pool screenings identified individual clones capable of supporting PCR reactions with the CCCKR-specific primers. One clone, 881F10, contained 640 kb of human genomic DNA from chromosome 3p21 including the genes for CCCKR1 and CCCKR2, as determined by PCR and hybridization. An overlapping YAC clone, 941A7, contained 700 kb of human genomic DNA and also contained the genes for CCCKR1 and CCCKR2. Consequently, further mapping studies were undertaken using these two YAC clones. Southern analyses revealed that CCCKR1 and CCCKR2 were located within approximately 100 kb of one another.

The close proximity of the CCCKR1 and CCCKR2 genes suggested that novel related genes might be linked to CCCKR1 and CCCKR2. Using DNA from yeast containing YAC clones 881F10 and 941A7 as templates, PCR reactions were performed to amplify any linked receptor genes. Degenerate oligodeoxyribonucleotides were designed as PCR primers. These oligonucleotides corresponded to regions encoding the second intracellular loop and the sixth transmembrane domain of CC chemokine receptors, as deduced from aligned sequence comparisons of CCCKR1,

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CCCKR2, and V28. V28 was used because it is an orphan receptor that exhibits the characteristics of a chemokine receptor; V28 has also been mapped to human chromosome 3. *Raport et al.*, Gene 163:295-299 (1995). Of further note, the two splice variants of CCCKR2, CCCKR2A and 5 CCCKR2B, are identical in the second intracellular loop and sixth transmembrane domain regions used in the analysis. The 5' primer, designated V28degf2, contains an internal *Bam*HI site (see below); its sequence is presented in SEQ ID NO:5. The sequence of primer V28degf2 corresponds to DNA encoding the second intracellular loop region of the 10 canonical receptor structure. See *Probst et al., supra*. The 3' primer, designated V28degr2, contains an internal *Hind*III site (see below); its sequence is presented in SEQ ID NO:6. The sequence of primer V28degr2 corresponds to DNA encoding the sixth transmembrane domain of the canonical receptor structure.

15               Amplified PCR DNA was subsequently digested with *Bam*HI and *Hind*III to generate fragments of approximately 390 bp, consistent with the fragment size predicted from inspection of the canonical sequence. Following endonuclease digestion, these PCR fragments were cloned into pBluescript (Stratagene Inc., LaJolla, CA). A total of 54 cloned fragments 20 were subjected to automated nucleotide sequence analyses. In addition to sequences from CCCKR1 and CCCKR2, sequences from the two novel chemokine receptor genes of the invention were identified. These two novel chemokine receptor genes were designated 88-2B and 88C.

25               Restriction endonuclease mapping and hybridization were utilized to map the relative positions of genes encoding the receptors 88C, 88-2B, CCCKR1, and CCCKR2. These four genes are closely linked, as the gene for 88C is approximately 18 KBP from the CCCKR2 gene on human chromosome 3p21.

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Example 2

Full-length 88-2B and 88C cDNAs were isolated from a macrophage cDNA library by the following procedure. Initially, a cDNA library, described in *Tjoelker et al.*, *Nature* 374:549-553 (1995), was constructed in pRc/CMV (Invitrogen Corp., San Diego, CA) from human macrophage mRNA. The cDNA library was screened for the presence of 88-2B and 88C cDNA clones by PCR using unique primer pairs corresponding to 88-2B or 88C. The PCR protocol involved an initial denaturation at 94°C for four minutes. Polynucleotides were then amplified using 33 cycles of PCR under the following conditions: Denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for two minutes. The first primer specific for 88-2B was primer 88-2B-f1, presented in SEQ ID NO:11. It corresponds to the sense strand of SEQ ID NO:3 at nucleotides 844-863. The second PCR primer specific for the gene encoding 88-2B was primer 88-2B-r1, presented in SEQ ID NO:12; the 88-2B-r1 sequence corresponds to the anti-sense strand of SEQ ID NO:3 at nucleotides 1023-1042. Similarly, the sequence of the first primer specific for the gene encoding 88C, primer 88C-f1, is presented in SEQ ID NO:13 and corresponds to the sense strand of SEQ ID NO:1 at nucleotides 453-471. The second primer specific for the gene encoding 88C is primer 88C-r3, presented in SEQ ID NO:14; the sequence of 88C-r3 corresponds to the anti-sense strand of SEQ ID NO:1 at nucleotides 744-763.

The screening identified clone 777, a cDNA clone of 88-2B. Clone 777 contained a DNA insert of 1915 bp including the full length coding sequence of 88-2B as determined by the following criteria: the clone contained a long open reading frame beginning with an ATG codon, exhibited a Kozak sequence, and had an in-frame stop codon upstream. The DNA and deduced amino acid sequences of the insert of clone 777 are presented in SEQ ID NO:3 and SEQ ID NO:4, respectively. The 88-2B transcript was relatively rare in the macrophage cDNA library. During the library screen, only three 88-2B clones were identified from an estimated total of three million clones.

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Screening for cDNA clones encoding the 88C chemokine receptor identified clones 101 and 134 which appeared to contain the entire 88C coding region, including a putative initiation codon. However, these clones lacked the additional 5' sequence needed to confirm the identity of the initiation codon. The 88C transcript was relatively abundant in the macrophage cDNA Library. During the library screen, it was estimated that 88C was present at one per 3000 transcripts (in a total of approximately three million clones in the library).

RACE PCR (Rapid Amplification of cDNA Ends) was performed to extend existing 88C clone sequences, thereby facilitating the accurate characterization of the 5' end of the 88C cDNA. Human spleen 5'-RACE-ready cDNA was purchased from Clontech Laboratories, Inc., Palo Alto, CA, and used according to the manufacturer's recommendations. The cDNA had been made "5'-RACE-ready" by ligating an anchor sequence to the 5' ends of the cDNA fragments. The anchor sequence is complementary to an anchor primer supplied by Clontech Laboratories, Inc., Palo Alto, CA. The anchor sequence-anchor primer duplex polynucleotide contains an *EcoRI* site. Human spleen cDNA was chosen as template DNA because Northern blots had revealed that 88C was expressed in this tissue. The PCR reactions were initiated by denaturing samples at 94°C for four minutes. Subsequently, sequences were amplified using 35 cycles involving denaturation at 94°C for one minute, annealing at 60°C for 45 seconds, and extension at 72°C for two minutes. The first round of PCR was performed on reaction mixtures containing 2 $\mu$ l of the 5'-RACE-ready spleen cDNA, 1  $\mu$ l of the anchor primer, and 1  $\mu$ l of primer 88c-r4 (100 ng/ $\mu$ l) in a total reaction volume of 50  $\mu$ l. The 88C-specific primer, primer 88c-r4 (5'-GATAAGCCTCACAG-CCCTGTG-3'), is presented in SEQ ID NO:7. The sequence of primer 88c-r4 corresponds to the anti-sense strand of SEQ ID NO:1 at nucleotides 745-765. A second round of PCR was performed on reaction mixtures including 1  $\mu$ l of the first PCR reaction with 1  $\mu$ l of anchor primer and 1  $\mu$ l of primer 88C-rlb (100 ng/ $\mu$ l) containing the following sequence

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(5'-GCTAAGCTT~~GATGACTAT~~CTTAATGTC-3') and presented in SEQ ID NO:8. The sequence of primer 88C-rib contains an internal *Hind*III cloning site (underlined). The sequence 3' of the *Hind*III site corresponds to the anti-sense strand of SEQ ID NO:1 at nucleotides 636-654. The resulting PCR product was digested with *Eco*RI and *Hind*III and fractionated on a 1% agarose gel. The approximately 700 bp fragment was isolated and cloned into pBluescript. Clones with the largest inserts were sequenced. Alternatively, the intact PCR product was ligated into vector pCR using a commercial TA cloning kit (Invitrogen Corp., San Diego, CA) for subsequent nucleotide sequence determinations.

The 88-2B and 88C cDNAs were sequenced using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin Elmer Corp., Foster City, CA) and an Applied Biosystems 373A DNA Sequencer. The insert of clone 777 provided the double-stranded template for sequencing reactions used to determine the 88-2B cDNA sequence. The sequence of the entire insert of clone 777 was determined and is presented as the 88-2B cDNA sequence and deduced amino acid sequence in SEQ ID NO:3. The sequence is 1915 bp in length, including 361 bp of 5' untranslated DNA (corresponding to SEQ ID NO:3 at nucleotides 1-361), a coding region of 1065 bp (corresponding to SEQ ID NO:3 at nucleotides 362-1426), and 489 bp of 3' untranslated DNA (corresponding to SEQ ID NO:3 at nucleotides 1427-1915). The 88-2B genomic DNA, described in Example 1 above, corresponds to SEQ ID NO:3 at nucleotides 746-1128. The 88C cDNA sequence, and deduced amino acid sequence, is presented in SEQ ID NO:1. The 88C cDNA sequence is a composite of sequences obtained from RACE-PCR cDNA, clone 134, and clone 101. The RACE-PCR cDNA was used as a sequencing template to determine nucleotides 1-654 in SEQ ID NO:1, including the unique identification of 9 bp of 5' untranslated cDNA sequence in SEQ ID NO:1 at nucleotides 1-9. The sequence obtained from the RACE PCR cDNA confirmed the position of the first methionine codon at nucleotides 55-57 in SEQ ID NO:1, and supported the conclusion that clone 134 and clone 101 contained full-length copies of the 88C coding region. Clone 134 contained

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45 bp of 5' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 10-54), the 1056 bp 88C coding region (corresponding to SEQ ID NO:1 at nucleotides 55-1110), and 492 bp of 3' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 1111-1602). Clone 101 contained 25 bp of 5' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 30-54), the 1056 bp 88C coding region (corresponding to SEQ ID NO:1 at nucleotides 55-1110), and 2273 bp of 3' untranslated cDNA (corresponding to SEQ ID NO:1 at nucleotides 1111-3383). The 88C genomic DNA described in Example 1 above, corresponds to SEQ ID NO:1 at nucleotides 424-809.

The deduced amino acid sequences of 88-2B and 88C revealed hydrophobicity profiles characteristic of GPCRs, including seven hydrophobic domains corresponding to GPCR transmembrane domains. Sequence comparisons with other GPCRs also revealed a degree of identity. Significantly, the deduced amino acid sequences of both 88-2B and 88C had highest identity with the sequences of the chemokine receptors. Table 1 presents the results of these amino acid sequence comparisons.

Table 1

Chemokine Receptors	88-2B	88C
IL-8RA	30%	30%
IL-8RB	31%	30%
CCCKR1	62%	54%
CCCKR2A	46%	66%
CCCKR2B	50%	72%
88-2B	100%	50%
88-C	50%	100%

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Table I shows that 88-2B is most similar to CCCKR1 (62% identical at the amino acid level) and 88C is most similar to CCCKR2 (72% identical at the amino acid level).

The deduced amino acid sequences of 88-2B and 88C also reveal the intracellular and extracellular domains characteristic of GPCRs. The 88-2B extracellular domains correspond to the amino acid sequence provided in SEQ ID NO:3, and SEQ ID NO:4, at amino acid residues 1-36, 93-107, 171-196, and 263-284. The extracellular domains of 88-2B are encoded by polynucleotide sequences corresponding to SEQ ID NO:3 at nucleotides 362-469, 638-682, 872-949, and 1148-1213. Extracellular domains of 88C include amino acid residues 1-32, 89-112, 166-191, and 259-280 in SEQ ID NO:1 and SEQ ID NO:2. The 88C extracellular domains are encoded by polynucleotide sequences that correspond to SEQ ID NO:1 at nucleotides 55-150, 319-390, 550-627, and 829-894. The intracellular domains of 88-2B include amino acids 60-71, 131-151, 219-240, and 306-355 of SEQ ID NO:3 and SEQ ID NO:4. Those domains are encoded by polynucleotide sequences corresponding to SEQ ID NO:3 at nucleotides 539-574, 752-814, 1016-1081, and 1277-1426, respectively. The 88C intracellular domains include amino acid residues 56-67, 125-145, 213-235, and 301-352 of SEQ ID NO:1 and SEQ ID NO:2. The intracellular domains of 88C are encoded by polynucleotide sequences corresponding to SEQ ID NO:1 at nucleotides 220-255, 427-489, 691-759, and 955-1110.

In addition, a macaque 88C DNA was amplified by PCR from macaque genomic DNA using primers corresponding to 5' and 3' flanking regions of the human 88C cDNA. The 5' primer corresponded to the region immediately upstream of and including the initiating Met codon. The 3' primer was complementary to the region immediately downstream of the termination codon. The primers included restriction sites for cloning into expression vectors. The sequence of the 5' primer was GACAAGCTTCACAGGGTGGAACAAAGATG (With the HindIII site underlined) (SEQ ID NO: 17) and the sequence of the 3' primer was GTCTCTAGACCACTTGAGTCCGTGTCA (with the XbaI site underlined) (SEQ ID

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NO: 18). The conditions of the PCR amplification were 94°C for eight minutes, then 40 cycles of 94°C for one minute, 55°C for forty-five seconds, and 72°C one minute. The amplified products were cloned into the HindIII and XbaI sites of pcDNA3 and a clone was obtained and sequenced. The full length macaque cDNA and deduced amino acid sequences are presented in SEQ ID NOs: 19 and 20, respectively. The nucleotide sequence of macaque 88C is 98% identical to the human 88C sequence. The deduced amino acid sequences are 97% identical.

Example 3

10 The mRNA expression patterns of 88-2B and 88C were determined by Northern blot analyses.

Northern blots containing immobilized poly A<sup>+</sup> RNA from a variety of human tissues were purchased from Clontech Laboratories, Inc., Palo Alto, CA. In particular, the following tissues were examined: heart, 15 brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes.

A probe specific for 88-2B nucleotide sequences was generated from cDNA clone 478. The cDNA insert in clone 478 contains sequence corresponding to SEQ ID NO: 3 at nucleotides 641-1915. To generate a 20 probe, clone 478 was digested and the insert DNA fragment was isolated following gel electrophoresis. The isolated insert fragment was then radiolabeled with <sup>32</sup>P-labeled nucleotides, using techniques known in the art.

A probe specific for 88C nucleotide sequences was generated by isolating and radiolabeling the insert DNA fragment found in clone 493. 25 The insert fragment from clone 493 contains sequence corresponding to SEQ ID NO: 1 at nucleotides 421-1359. Again, conventional techniques involving <sup>32</sup>P-labeled nucleotides were used to generate the probe.

Northern blots probed with 88-2B revealed an approximately 1.8 kb mRNA in peripheral blood leukocytes. The 88C Northerns showed an 30 approximately 4 kb mRNA in several human tissues, including a strong signal when probing spleen or thymus tissue and less intense signals when analyzing

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mRNA from peripheral blood leukocytes and small intestine. A relatively weak signal for 88C was detected in lung tissue and in ovarian tissue.

The expression of 88C in human T-cells and in hematopoietic cell lines was also determined by Northern blot analysis. Levels of 88C in CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were very high. The transcript was present at relatively high levels in myeloid cell lines THP1 and HL-60 and also found in the B cell line Jijoye. In addition, the cDNA was a relatively abundant transcript in a human macrophage cDNA library based on PCR amplification of library subfractions.

10

#### Example 4

The 88-2B and 88C cDNAs were expressed by recombinant methods in mammalian cells.

For transient transfection experiments, 88C was subcloned into the mammalian cell expression vector pBJ1 (Ishi, K. et. al., *J. Biol. Chem* 270:16435-16440 (1995). The construct included sequences encoding a prolactin signal sequence for efficient cell surface expression and a FLAG epitope at the amino terminus of 88C to facilitate detection of the expressed protein. The FLAG epitope consists of the sequence "DYKDDDD." COS-7 cells were transiently transfected with the 88C expression plasmid using Lipofectamine (Life Technology, Inc., Grand Island, NY) following the manufacturer's instructions. Briefly, cells were seeded in 24-well plates at a density of 4 X 10<sup>4</sup> cells per well and grown overnight. The cells were then washed with PBS, and 0.3 mg of DNA mixed with 1.5 µl of lipofectamine in 0.25 ml of Opti-MEM was added to each well. After 5 hours at 37°C, the medium was replaced with medium containing 10% FCS. quantitative ELISA confirmed that 88C was expressed at the cell surface in transiently transfected COS-7 cells using the M1 antibody specific for the FLAG epitope (Eastman Co., New Haven, CT).

The FLAG-tagged 88C receptor was also stably transfected into HEK-293 cells, a human embryonic kidney cell line, using transfection reagent DOTAP (N-[1-[(2,3-Dioleyloxy)propyl]-N,N,N-trimethyl-

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ammoniummethylsulfate, Boehringer-Mannheim, Inc., Indianapolis, IN) according to the manufacturer's recommendations. Stable lines were selected in the presence of the drug G418. The transfected HEK-293 cells were evaluated for expression of 88C at the cell surface by ELISA, using the M1 antibody to the FLAG epitope. ELISA showed that 88C tagged with the FLAG epitope was expressed at the cell surface of stably transformed HEK-293 Cells.

The 88-2B and 88C cDNAs were used to make stable HEK-293 transfectants. The 88-2B receptor cDNA was cloned behind the cytomegalovirus promoter in pRc/CMV (Invitrogen Corp., San Diego, CA) using a PCR-based strategy. The template for the PCR reaction was the cDNA insert in clone 777. The PCR primers were 88-2B-3 (containing an internal *Xba*I site) and 88-2B-5 (containing an internal *Hind*III site). The nucleotide sequence of primer 88-2B-3 is presented in SEQ ID NO:9; the nucleotide sequence of primer 88-2B-5 is presented in SEQ ID NO:10. An 1104 bp region of cDNA was amplified. Following amplification, the DNA was digested with *Xba*I and *Hind*III and cloned into similarly digested pRc/CMV. The resulting plasmid was named 777XP2, which contains 18 bp of 5' untranslated sequence, the entire coding region of 88-2B, and 3 bp of 3' untranslated sequence. For the 88C sequence, the full-length cDNA insert in clone 134 was not further modified before transfecting HEK-293 cells.

To create stably transformed cell lines, the pRc/CMV recombinant clones were transfected using transfection reagent DOTAP (N-[1-[ (2,3-Dioleyloxy)propyl]-N,N,N-trimethyl-  
25 ammoniummethylsulfate, Boehringer-Mannheim, Inc., Indianapolis, IN) according to the manufacturer's recommendations, into HEK-293 cells, a human embryonic kidney cell line. Stable lines were selected in the presence of the drug G418. Standard screening procedures (*i.e.*, Northern blot analyses) were performed to identify stable cell lines expressing the highest levels of 88-2B and 88C mRNA.

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Example 5

A. Ca<sup>++</sup> Flux Assays

To analyze polypeptide expression, a functional assay for chemokine receptor activity was employed. A common feature of signalling through the known chemokine receptors is that signal transduction is associated with the release of intracellular calcium cations. Therefore, intracellular Ca<sup>++</sup> concentration in the transfected HEK-293 cells was assayed to determine whether the 88-2B or 88C receptors responded to any of the known chemokines.

HEK-293 cells, stably transfected with 88-2B, 88C (without the FLAG epitope sequence), or a control coding region (encoding IL8R or CCCKR2, see below) as described above, were grown in T75 flasks to approximately 90% confluence in MEM + 10% serum. Cells were then washed, harvested with versene (0.6 mM EDTA, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.14 M NaCl, 3 mM KCl, and 1 mM glucose), and incubated in MEM + 10% serum + 1 μM Fura-2 AM (Molecular Probes, Inc., Eugene, OR) for 30 minutes at room temperature. Fura-2 AM is a Ca<sup>++</sup>-sensitive dye. The cells were resuspended in Dulbecco's phosphate-buffered saline containing 0.9 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> (D-PBS) to a concentration of approximately 10<sup>7</sup> cells/ml and changes in fluorescence were monitored using a fluorescence spectrophotometer (Hitachi Model F-4010). Approximately 10<sup>6</sup> cells were suspended in 1.8 ml D-PBS in a cuvette maintained at 37°C. Excitation wavelengths alternated between 340 and 380 nm at 4 second intervals; the emission wavelength was 510 nm. Test compositions were added to the cuvette via an injection port; maximal Ca<sup>++</sup> flux was measured upon the addition of ionomycin.

Positive responses were observed in cells expressing IL-8RA when stimulated with IL-8 and also when CCCKR2 was stimulated with MCP-1 or MCP-3. However, HEK-293 cells expressing either 88-2B or 88C failed to show a flux in intracellular Ca<sup>++</sup> concentration when exposed to any of the following chemokines: MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β, IL8,

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NAP-2, gro/MGSA, IP-10, ENA-78, or PF-4. (Peprotech, Inc., Rocky Hill, NJ).

Using a more sensitive assay, a  $\text{Ca}^{++}$  flux response to RANTES was observed microscopically in Fura-2 AM-loaded cells expressing 88-2B.

5      The assay involved cells and reagents prepared as described above. RANTES (Regulated on Activation, Normal T Expressed and Secreted) is a CC chemokine that has been identified as a chemoattractant and activator of eosinophils. See Neote *et al.*, *supra*. This chemokine also mediates the release of histamine by basophils and has been shown to function as a chemoattractant for memory T cells *in vitro*. Modulation of 88-2B receptor activities is therefore contemplated to be useful in modulating leukocyte activation.

10

FLAG tagged 88C receptor was expressed in HEK-293 cells and tested for chemokine interactions in the  $\text{Ca}^{++}$  flux assay. Cell surface expression of 88C was confirmed by ELISA and by FACScan analysis using the M1 antibody. The chemokines RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  all induced a  $\text{Ca}^{++}$  flux in 88C-transfected cells when added at a concentration of 100 nM.

20       $\text{Ca}^{++}$  flux assays can also be designed to identify modulators of chemokine receptor binding. The preceding fluorimetric or microscopic assays are carried out in the presence of test compounds. If  $\text{Ca}^{++}$  flux is increased in the presence of a test compound, that compound is an activator of chemokine receptor binding. In contrast, a diminished  $\text{Ca}^{++}$  flux identifies the test compound as an inhibitor of chemokine receptor binding.

25      B.      Phosphoinositol Hydrolysis

Another assay for ligands or modulators involves monitoring phospholipase C activity, as described in Hung *et al.*, *J. Biol. Chem.* 116:827-832 (1992). Initially, host cells expressing a chemokine receptor are loaded with  $^3\text{H}$ -inositol for 24 hours. Test compounds (*i.e.*, potential ligands) are 30 then added to the cells and incubated at 37°C for 15 minutes. The cells are then exposed to 20 mM formic acid to solubilize and extract hydrolyzed

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metabolites of phosphoinositol metabolism (*i.e.*, the products of phospholipase C-mediated hydrolysis). The extract is subjected to anion exchange chromatography using an AG1X8 anion exchange column (formate form). Inositol phosphates are eluted with 2 M ammonium formate/0.1 M formic acid and the <sup>3</sup>H associated with the compounds is determined using liquid scintillation spectrophotometry. The phospholipase C assay can also be exploited to identify modulators of chemokine receptor activity. The aforementioned assay is performed as described, but with the addition of a potential modulator. Elevated levels of detectable label would indicate the modulator is an activator; depressed levels of the label would indicate the modulator is an inhibitor of chemokine receptor activity.

The phospholipase C assay was performed to identify chemokine ligands of the FLAG-tagged 88C receptor. Approximately 24 hours after transfection, COS-7 cells expressing 88C were labeled for 20-24 hours with *myo*-[2-<sup>3</sup>H]inositol (1  $\mu$ Ci/ml) in inositol-free medium containing 10% dialyzed FCS. Labeled cells were washed with inositol-free DMEM containing 10 mM LiCl and incubated at 37°C for 1 hour with inositol-free DMEM containing 10 mM LiCl and one of the following chemokines: RANTES, MIP-1 $\beta$ , MIP-1 $\alpha$ , MCP-1, IL-8, or the murine MCP-1 homolog JE. Inositol phosphate (IP) formation was assayed as described in the previous paragraph. After incubation with chemokines, the medium was aspirated and cells were lysed by addition of 0.75 ml of ice-cold 20 mM formic acid (30 min). Supernatant fractions were loaded onto AG1-X8 Dowex columns (Biorad, Hercules, CA), followed by immediate addition of 3 ml of 50 mM NH<sub>4</sub>OH. The columns were then washed with 4 ml of 40 mM ammonium formate, followed by elution with 2 M ammonium formate. Total inositol phosphates were quantitated by counting beta-emissions.

Because it has been shown that some chemokine receptors, such as IL8RA AND IL8RB, require cotransfection with an exogenous G protein before signalling can be detected in COS-7 cells, the 88C receptor was co-expressed with the chimeric G protein Gqi5 (Conklin, *et al.*, *Nature* 363:274-276, (1993). Gqi5 is a G protein which has the carboxyl terminal five amino

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acids of Gi (which bind to the receptor) spliced onto G $\alpha$ q. Co-transfection with Gqi5 significantly potentiates signaling by CCCKR1 and CCKR2B. Co-transfection with Gqi5 revealed that 88C signaled well in response to RANTES, MIP-1 $\beta$ , and MIP-1 $\alpha$ , but not in response to MCP-1, IL-8 or the 5 murine MCP-1 homologue JE. Dose-response curves revealed EC<sub>50</sub> values of 1nM for RANTES, 6nM for MIP-1 $\beta$ , and 22nM for MIP-1 $\alpha$ .

88C is the first cloned human receptor with a signaling response to MIP-1 $\beta$ . Compared with other CC chemokines, MIP-1 $\beta$  clearly has a unique cellular activation pattern. It appears to activate T cells but not 10 monocytes (Baggiolini et al., Supra) which is consistent with receptor stimulation studies. For example, while MIP-1 $\beta$  binds to CCCKR1, it does not induce calcium flux (Neote et al., Supra). In contrast, MIP-1 $\alpha$  and RANTES bind to and causes signalling in CCCKR1 and CCCKR5 (RANTES also causes activation of CCCKR3). MIP-1 $\beta$  thus appears to be much more 15 selective than other chemokines of the CC chemokine family. Such selectivity is of therapeutic significance because a specific beneficial activity can be stimulated (such as suppression of HIV infection) without stimulating multiple leukocyte populations which results in general pro-inflammatory activities.

#### C. BINDING ASSAYS

Another assay for receptor interaction with chemokines was a 20 modification of the binding assay described by Ernst et al. *J. Immunol.* 152:3541-3549 (1994). MIP-1 $\beta$  was labeled using the Bolton and Hunter reagent (di-iodide, NEN, Wilmington, DE), according to the manufacturer's instructions. Unconjugated iodide was separated from labeled protein by 25 elution using a PD-10 column (Pharmacia) equilibrated with PBS and BSA (1% w/v). The specific activity was typically 2200 Ci/mmole. Equilibrium binding was performed by adding <sup>125</sup>I-labeled ligand with or without a 100-fold excess of unlabeled ligand, to 5 X 10<sup>5</sup> HEK-293 cells transfected with 30 88C tagged with the FLAG epitope in polypropylene tubes in a total volume of 300  $\mu$ l (50 mM HEPES pH 7.4, 1 mM CaCl<sub>2</sub>, MgCl<sub>2</sub>, 0.5 % BSA) and incubating for 90 minutes at 27°C with shaking at 150 rpm. The cells were

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collected, using a Skatron cell harvester (Skatron Instruments Inc., Sterling, VA), on glass fiber filters presoaked in 0.3% polyethyleneimine and 0.2% BSA. After washing, the filters were removed and bound ligand was quantitated by counting gamma emissions. Ligand binding by competition with unlabeled ligand was determined by incubation of  $5 \times 10^5$  transfected cells (as above) with 1.5 nM of radiolabeled ligand and the indicated concentrations of unlabeled ligand. The samples were collected, washed and counted as above. The data was analyzed using the curve-fitting program Prism (GraphPad Inc., San Diego, CA) and the iterative non-linear regression program, LIGAND (PM220).

In equilibrium binding assays,  $^{88}\text{C}$  receptor bound radiolabeled MIP-1 $\beta$  in a specific and saturable manner. Analysis of this binding data by the method of Scatchard revealed a dissociation constant ( $K_d$ ) of 1.6 nM. Competition binding assays using labeled MIP-1 $\beta$  revealed high-affinity binding of MIP-1 $\beta$  ( $IC_{50} = 7.4$  nM), RANTES ( $IC_{50} = 6.9$  nM), and MIP-1 $\alpha$  ( $IC_{50} = 7.4$  nM), consistent with the signaling data obtained in transiently transfected COS-7 cells as discussed in section B above.

### Example 6

The chemokines MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES have been shown to inhibit replication of HIV-1 and HIV-2 in human peripheral blood mononuclear cells and PM1 cells (*Cocchi, et. al., supra*). In view of this finding and in view of the results described in Example 5, the present invention contemplates that activation of or ligand binding to the 88C receptor may provide a protective role in HIV infection.

Recently, it has been reported that the orphan G protein-coupled receptor, fusin, can act as a co-receptor for HIV entry. Fusin/CXCR4 in combination with CD4, the primary HIV receptor, apparently facilitates HIV infection of cultured T cells (*Feng, et al., Science* 272:872-877 (1996). Based upon the homology of fusin to chemokine receptors and the chemokine binding profile of 88C, and because 88C is constitutively expressed in T cells

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and abundantly expressed in macrophages, 88C is likely to be involved in viral and HIV infection.

The function of 88C and 88-2B as co-receptors for HIV was determined by transfecting cells which express CD4 with 88C or 88-2B and challenging the co-transfected cells with HIV. Only cells expressing both CD4 and a functional co-receptor for HIV become infected. HIV infection can be determined by several methods. ELISAs which test for expression of HIV antigens are commercially available, for example Coulter HIV-1 p24 antigen assay (US Patent Nos. 4,886,742), Coulter Corp., 11800 SW 147th Ave., Miami, FL 33196. Alternatively, the test cells can be engineered to express a reporter gene such as *LACZ* attached to the HIV LTR promoter [Kimpton *et al.*, *J. Virol.* 66:2232-2239 (1992)]. In this method, cells that are infected with HIV are detected by a colorimetric assay.

88C was transiently transfected into a cat cell line, CCC [Clapham, *et al.*, *181*:703-715 (1991)], which had been stably transformed to express human CD4 (CCC-CD4). These cells are normally resistant to infection by any strain of HIV-1 because they do not endogenously express 88C. In these experiments, CCC/CD4 cells were transiently transfected with 88C cloned into the expression vector pcDNA3.1 (Invitrogen Corp., San Diego, CA) using lipofectamine (Gibco BRL, Gaithersburg, MD). Two days after transfection, cells were challenged with HIV. After 4 days of incubation, cells were fixed and stained for p24 antigen as a measure of HIV infection. 88C expression by these cells rendered them susceptible to infection by several strains of HIV-1. These strains included four primary non-syncytium-inducing HIV-1 isolates (M23, E80, SL-2 and SF-162) which were shown to use only 88C as a co-receptor but not fusin. Several primary syncytium-inducing strains of HIV-1 (2006, M13, 2028 and 2076) used either 88C or fusin as a co-receptor. Also, two established clonal HIV-1 viruses (GUN-1 and 89.6) used either 88C or fusin as a co-receptor.

It has been reported that some strains of HIV-2 can infect certain CD4-negative cell lines, thus implying a direct interaction of HIV-2 with a receptor other than CD4 [Clapham, *et al.*, *J. Virol.* 66:3531-3537

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(1992)] For some strains of HIV-2, this infection is facilitated by the presence of soluble CD4 (sCD4). Since 88-2B shares high sequence similarity with other chemokine receptors that act as HIV co-receptors (namely 88C and fusin), 88-2B was considered to be a likely HIV-2 co-receptor. The role of 5 88-2B as an HIV-2 co-receptor was demonstrated using HIV-2 strain ROD/B. Cat CCC cells which do not endogeneously express CD4 were transfected with 88-2B. In these experiments, cells were transfected with pcDNA3.1 containing 88-2B using lipofectamine and infected with HIV-2 48 hours later. Three days after infection, cells were immunostained for the presence of HIV-10 2 envelope glycoproteins. The presence of sCD4 during HIV-2<sub>ROD/B</sub> challenge increased the infection of these cells by 10-fold. The entry of HIV-2 into the 88-2B transfected cells could be blocked by the presence of 400-800 ng/ml eotaxin, one of the ligands for 88-2B. The baseline infectivity levels of CCC/88-2B (with no soluble CD4) were equivalent to CCC cells which were 15 not transfected with 88-2B.

The role of 88-2B and 88C as co-receptors for HIV was confirmed by preparing and challenging cell lines stably transformed to express 88C or 88-2B with various strains of HIV and SIV. These results are described in Example 7.

20 Alternatively, the co-receptor role of 88C and 88-2B can be demonstrated by an experimental method which does not require the use of live virus. In this method, cell lines co-expressing 88C or 88-2B, CD4 and a LACZ reporter gene are mixed with a cell line co-expressing the HIV envelope glycoprotein (ENV) and a transcription factor for the reporter gene 25 construct (Nussbaum, *et al.*, 1994 *J. Virol.* 68:5411). Cells expressing a functional co-receptor for HIV will fuse with the ENV expressing cells and thereby allow expression of the reporter gene. In this method, detection of reporter gene product by colorimetric assay indicates that 88C or 88-2B function as a co-receptor for HIV.

30 The mechanism by which chemokines inhibit viral infection has not yet been elucidated. One possible mechanism involves activation of the receptor by binding of a chemokine. The binding of the chemokine leads to

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signal transduction events in the cell that renders the cell resistant to viral infection and/or prevents replication of the virus in the cell. Similar to interferon induction, the cell may differentiate such that it is resistant to viral infection, or an antiviral state is established. Alternatively, a second mechanism involves direct interference with viral entry into cells by blocking access of viral envelope glycoproteins to the co-receptor by chemokine binding. In this mechanism, G-protein signalling is not required for chemokine suppression of HIV infection.

To distinguish between two mechanisms by which 88C or 88-2B may function as co-receptors for viral or HIV infection, chemokine binding to the receptor is uncoupled from signal transduction and the effect of the chemokine on suppression of viral infection is determined.

Ligand binding can be uncoupled from signal transduction by the addition of compounds which inhibit G-protein mediated signaling. These compounds include, for example, pertussis toxin and cholera toxin. In addition, downstream effector polypeptides can be inhibited by other compounds such as wortmannin. If G-protein signalling is involved in suppression of viral infection, the addition of such compounds would prevent suppression of viral infection by the chemokine. Alternatively, key residues or receptor domains of 88C or 88-2B receptor required for G-protein coupling can be altered or deleted such that G-protein coupling is altered or destroyed but chemokine binding is not affected.

Under these conditions, if chemokines are unable to suppress viral or HIV infection, then signaling through a G-protein is required for suppression of viral or HIV infection. If however, chemokines are able to suppress viral infection, then G-protein signaling is not required for chemokine suppression of viral infection and the protective effects of chemokines may be due to the chemokine blocking the availability of the receptor for the virus.

Another approach involves the use of antibodies directed against 88C or 88-2B. Antibodies which bind to 88C or 88-2B which can be shown not to elicit G-protein signaling may block access to the chemokine or viral

binding site of the receptor. If in the presence of antibodies to 88C or 88-2B, viral infection is suppressed, then the mechanism of the protective effects of chemokines is blocking viral access to its receptor. Feng, *et al.* Reported that antibodies to the amino terminus of the fusin receptor suppressed HIV infection (Feng, *et al.*, 1996).

**Example 7**

Cell lines were stably transformed with 88C or 88-2B to further delineate the role of 88C and 88-2B in HIV infection. Kimpton and Emerman, "Detection of Replication-Competent and Pseudotyped Human Immunodeficiency Virus with a Sensitive Cell Line on the Basis of Activation of an Integrated Beta-Galactosidase Gene," *J. Virol.*, 66(5):3026-3031 (1992) previously described an indicator cell line, herein identified as HeLa-MAGI cells. HeLa-MAGI cells are HeLa cells that have been stably transformed to express CD4 as well as integrated HIV-1 LTR which drives expression of a nuclear localized  $\beta$ -galactosidase gene. Integration of an HIV provirus in the cells leads to production of the viral transactivator, Tat, which then turns on expression of the  $\beta$ -galactosidase gene. The number of cells that stain positive with X-gal for  $\beta$ -galactosidase activity *in situ* is directly proportional to the number of infected cells.

These HeLa-MAGI cells can detect lab-adapted isolates of HIV-1 but only a minority of primary isolates [Kimpton and Emerman, *supra*], and cannot detect most SIV isolates [Chackerian *et al.*, "Characterization of a CD4-Expressing Macaque Cell Line that can Detect Virus After A Single Replication Cycle and can be infected by Diverse Simian Immunodeficiency Virus Isolates." *Virology*, 213(2):6499-6505 (1995)].

In addition, Harrington and Geballe, "Co-Factor Requirement for Human Immunodeficiency Virus Type 1 Entry into a CD4-Expressing Human Cell Line, *J. Virol.*, 67:5939-5947 (1993) described a cell line based on U373 cells that had been engineered to express CD4 and the same LTR- $\beta$ -galactosidase construct. It was previously shown that this cell line, herein identified as U373-MAGI, could not be infected with any HIV (M or T-tropic)

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strain of HIV, but could be rendered susceptible to infection by fusion with HeLa cells [Harrington and Geballe, *supra*].

In order to construct indicator cell lines that could detect either Macrophage or T cell tropic viruses, epitope-tagged 88C or 88-2B encoding DNA was transfected into HeLa-MAGI or U373-MAGI cells by infection with a retroviral vector to generate HeLA-MAGI-88C or U373-MAGI-88C cell lines, respectively. Expression of the co-receptors on the cell surface was demonstrated by immunostaining live cells using the anti-FLAG M1 antibody and by RT-PCR.

The 88C and 88-2B genes utilized to construct HeLa-MAGI-88C and U373-MAGI-88C included sequences encoding the prolactin signal peptide followed by a FLAG epitope as described in Example 4. This gene was inserted into the retroviral vector pBabe-Puro [Morgenstern and Land *Nucleic Acids Research*, 18(12):3587-3596 (1990)]. High titer retroviral vector stocks pseudotyped with the VSV-G protein were made by transient transfection as described in Bartx *et al.*, *J. Virol.* 70:2324-2331 (1996), and used to infect HeLa-MAGI and U373-MAGI cells. Cells resistant to 0.6 µg/ml puromycin (HeLa) or 1 µg/ml puromycin (U373) were pooled. Each pool contained at least 1000 independent transduction events. An early passage (passage 2) stock of the original HeLa-MAGI cells [Kimpton and Emerman, *supra*] was used to create HeLa-MAGI-88C cells.

Infections of the indicator cell lines with HIV were performed in 12-well plates with 10-fold serial dilutions of 300 µl of virus in the presence of 30 µg/ml DEAE-Dextran as described [Kimpton and Emerman, *supra*].

All HIV-1 strains and SIV<sub>mac239</sub> were all obtained from the NIH AIDS Reference and Reagent Program. Molecular clones of primary HIV-2<sub>7312A</sub> [Gao *et al.*, "Genetic Diversity of Human Immunodeficiency Virus Type 2: Evidence for Distinct Sequence Subtypes with Differences in Virus Biology," *J. Virol.*, 68(11):7433-7447 (1992)] and SIVsmPbj1.9 [Dewhurst *et al.*, "Sequence Analysis and Acute Pathogenicity of Molecularly Cloned SIV<sub>smn</sub>-PBj14," *Nature*, 345:636-640 (1990)] were obtained from B. Hahn

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(UAB). All other SIV<sub>mac</sub> isolates were obtained from Julie Overbaugh (U. Washington, Seattle). Stocks from cloned proviruses were made by transient transfection of 293 cells. Other viral stocks were made by passage of virus in human peripheral blood mononuclear cells or in CEMx174 cells (for SIV stocks.) Viral stocks were normalized by ELISA or p24<sup>gag</sup> (Coulter Immunology) or p27<sup>gag</sup> (Coulter Immunology) for HIV-1 and HIV-2/SIV, respectively, using standards provided by the manufacturer.

U373-MAGI-88C cells and U373-MAGI cells (controls) and were infected with limiting dilutions of a T-tropic strain of HIV-1 (HIV<sub>LAI</sub>), an M-tropic strain (HIV<sub>YU-2</sub>), and an SIV isolate, SIV<sub>MAC</sub>239. Infectivity was measured by counting the number of blue cells per well per volume of virus (Table 2).

Table 2

	<u>virus strain<sup>a</sup></u>	<u>titer on cell line (IU/ml)<sup>b</sup></u>	
		U373-MAGI	U373-MAGI-88C
15	HIV-1 <sub>LAI</sub>	< 100	< 100
	HIV-1 <sub>YU-2</sub>	< 100	2.2 x 10 <sup>6</sup>
	SIV <sub>MAC</sub> 239	1.2 x 10 <sup>3</sup>	4 x 10 <sup>5</sup>

<sup>a</sup> Viruses derived by transfection of molecular clones into 293 cells.

20 <sup>b</sup> Infectious units (IU) per ml is the number of blue cells per well multiplied by the dilution of virus supernatant and normalized to 1 ml final volume.

Two days after infection, cells were fixed and stained for  $\beta$ -galactosidase activity with X-gal. The U373-derived MAGI cells were stained for 120 minutes at 37°C and the HeLa-derived MAGI cells were stained for 50 minutes at 37°C. Background staining of non-infected cells never exceeded more than approximately three blue cells per well. Only dark blue cells were counted, and syncytium with multiple nuclei were counted as a single infected cell. The infectious titer is the number of blue cells per well multiplied by the dilution of virus and normalized to 1 ml. The titer of

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HIV<sub>YU.2</sub> on U373-MAGI-88C cells was  $2 \times 10^6$ . In contrast, the titer of HIV-1<sub>LAI</sub> was less than 100 on U373-MAGI-88C. Thus, the specificity of a particular HIV strain for 88C varied by four orders of magnitude.

Although SIV<sub>MAC</sub>239 infection was increased to  $4 \times 10^5$  in  
5 U373-MAGI-88C it also clearly infected U373-MAGI cells (Table 2).

Next, a series of primary uncloned HIV strains and cloned M-tropic strains of HIV-1 were analyzed for their ability to infect indicator cell lines that express 88C.

As described above, HeLa-MAGI and HeLa-MAGI-88C cells  
10 were infected with limiting dilutions of various HIV strains. The two cloned M-tropic viruses, HIV<sub>JR-CSF</sub> and HIV<sub>YU.2</sub>, both infected HeLa-MAGI-88C, but not HeLa-MAGI cells, showing that both strains use 88C as a co-receptor (Table 3. See note c). However, a great disparity in the ability of each of these two viral strains to infect HeLa-MAGI-88C cells was observed,  $6.2 \times 15 10^5$  IU/ml for HIV<sub>YU.2</sub> and  $1.2 \times 10^4$  for HIV<sub>JR-CSF</sub>. The infectivity of virus stock (Table 3) is the number of infectious units per physical particle (represented here by the amount of viral core protein). In addition, it was observed that the infectivity of these two cloned viral strains differed by over 50-fold in viral stocks that were independently prepared.

20 The variability of infectivity of primary viral isolates was further examined by analyzing a collection of twelve different uncloned virus stocks from three different clades (Table 3). Three clade A primary isolates, three clade E isolates, and three additional clade B isolates from geographically diverse origins were used. With all nine strains, the primary 25 strains of HIV could be detected on HeLa-MAGI-88C cells, but not on HeLa-MAGI cells (Table 3). However, the efficacy of infection varied from five infectious units per ng p24 <sup>gag</sup> to over 100 infectious units per ng p24 <sup>gag</sup> (table 3). These results indicate that absolute infectivity of M-tropic strains varies considerably and is independent of clade. A hypothesis that may explain this 30 discrepancy may involve the affinity of the V3 loop of each viral strain for 88C after CD4 binding [Trkola *et al.*, *Nature*, 384(6605):184-187 (1996); Wu *et al.*, *Nature*, 384(6605):179-183 (1996)].

Table 3

<u>virus strain<sup>a</sup></u>	<u>viral sub-type (country of origin)<sup>b</sup></u>	<u>titer (IU/ml) on HeLa-MAGI-88C<sup>c</sup></u>	<u>P24<sup>gag</sup> ng/ml</u>	<u>Infectivity<sup>d</sup></u>
HIV-1 <sub>YU-2</sub>	B (USA)	6.2 x 10 <sup>3</sup>	2200	281
HIV-1 <sub>JR-CSF</sub>	B (USA)	12000	2800	4.2
5	HIV-1 <sub>TH020</sub>	E (Thailand)	4133	93
	HIV-1 <sub>TH021</sub>	E (Thailand)	4967	52
	HIV-1 <sub>TH022</sub>	E (Thailand)	200	15
	HIV-1 <sub>US660</sub>	B (USA)	2367	127
	HIV-1 <sub>UG031</sub>	A (Uganda)	1633	71
	HIV-1 <sub>RW009</sub>	A (Rwanda)	3333	158
	HIV-1 <sub>RW026</sub>	A (Rwanda)	739	143
	HIV-1 <sub>US727</sub>	B (USA)	14.067	289
	HIV-1 <sub>US954</sub>	B (USA)	5833	284
	HIV-1 <sub>LAI</sub>	B (France)	2.8 x 10 <sup>5</sup>	167
				1600

15           <sup>a</sup> HIV-1<sub>YU-2</sub> and HIV-1<sub>JR-CSF</sub> were derived by transfection of molecular clones. All others were tested as crude supernatants of uncloned viral stocks derived from infection of heterologous peripheral blood mononuclear cells.

20           <sup>b</sup> Clade designation according to Myers *et al.*, 1995 for the env gene; country of origin refers to the country of residence of the HIV-positive individual from whom blood was obtained for viral isolation (World Health Organization Viral Isolate Program).

25           <sup>c</sup> Infectious units (IU) per ml is the number of blue cells per well multiplied by the dilution of virus supernatant and normalized to 1 ml final volume. All viruses, except HIV-1<sub>LAI</sub>, had less than 10 IU/ml when tested on HeLa-MAGI cells without 88C. HIV<sub>LAI</sub>, a T-tropic strain, has a titer of 2.8 x 10<sup>5</sup> on HeLa-MAGI cells with or without 88C.

30           <sup>d</sup> Infectivity is the infectious units per ng P24<sup>gag</sup> (column four divided by column five).

The ability of the HeLa-MAGI-88C cells to detect HIV-2 and other SIV strains was also determined. HIV-2<sub>Rod</sub> has been reported to use fusin as a receptor even in the absence of CD4 [Endres *et al.*, *Cell*,

87(4):745-756 (1996)]. HIV-2<sub>Rod</sub> is able to infect HeLa-MAGI cells, however its infectivity is enhanced at least 10-fold in HeLa-Magi-88C (Table 4). HeLa cells endogenously express fusin. Thus, the molecular clone of HIV-2<sub>Rod</sub> is dual tropic, and is able to use 88C as one of its co-receptors in addition to CXCR4. Similarly, a primary strain of HIV-2<sub>7312A</sub> infected HeLa-MAGI-88C cells and not the HeLa-MAGI cells, indicating that like primary strain of HIV-1, it uses 88C as a receptor.

Table 4

virus strain <sup>a</sup>	reference	titer (IU/ml) on HeLa-MAGI <sup>b</sup>	titer (IU/ml) on HeLa-MAGI- 88C	Infectivity on HeLa- MAGI-88C <sup>c</sup>
HIV-2 <sub>Rod</sub>	(Guyader <i>et al.</i> , 1987)	967	5900	13
	HIV-2 <sub>7312A</sub>	<30	6500	17
	SIV <sub>MAC-239</sub>	<30	20900	90
	SIV <sub>MNE-C18</sub>	<30	15700	19
	SIV <sub>MNE-170</sub>	<30	10700	27
	SIV <sub>SM-Pbj1.9</sub>	<30	776	ND <sup>d</sup>
	SIV <sub>AGM-9063</sub>	<30	50	<1

<sup>a</sup> HIV-2 stocks. SIV<sub>SM-Pbj1.9</sub>, and SIV<sub>AGM-9063</sub> were tested directly after by transfection of molecular clones in 293 cells. All others were derived from transfection of molecular clones and subsequently amplified in CEMx174 cells.

<sup>b</sup> Infectious units (IU) per ml is the number of blue cells per well multiplied by the dilution of virus supernatant and normalized to 1 ml final volume. All viruses in this panel were also negative on HeLa-MAGI-88-2B.

<sup>c</sup> Infectivity is the infectious units (on 88C expressing cells) per ng P27<sup>env</sup> determined by ELISA.

<sup>d</sup> ND, not determined.

None of the SIV strains tested infected the HeLa-MAGI cells (Table 4), and none infected HeLa-MAGI cells that expresses 88-2B. This indicates that an alternative co-receptor used by SIV in U373 cells is not expressed in HeLa cells, and is not 88-2B. All SIV strains tested infected the

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HeLa-MAGI-88C cells to some extent (Table 3) indicating that all of the tested SIV strains use at least 88C as one of their co-receptors.

The classification of M-tropic and T-tropic strains of HIV in the past has often been correlated with another designation "non-syncytium inducing" (NSI), and "syncytium inducing" (SI), respectively. Assays based on the cell lines described herein are sensitive to syncytium formation. The infected cells can form large and small foci of infection containing multiple nuclei [Kimpton and Emerman, *supra*].

Experiments using multiple different viral strains and U373-MAGI-88C or HeLa-MAGI-88C indicate that SI/NSI designation is not meaningful because all viral strains formed syncytia if the correct co-receptor was present. These experiments show that syncytium formation is more likely a marker for the presence of an appropriate co-receptor on the infected cell, rather than an indication of tropism. Infection of the HeLa-MAGI-88C cells with SIV strains reported in the literature to be non-syncytium forming strains, in particular, SIV<sub>MAC</sub>239, SIV<sub>MNE</sub>C18, and SIV<sub>MNE</sub>170, was remarkable because the size of the syncytia induced in the monolayer was much larger than those induced by any other the HIV strains.

#### Example 8

Mouse monoclonal antibodies which specifically recognize 88C were prepared. The antibodies were produced by immunizing mice with a peptide corresponding to the amino terminal twenty amino acids of 88C. The peptide was conjugated to Keyhole Limpet Cyanin (KLH) according to the manufacturer's directions (Pierce, Imject maleimide activated KLH), emulsified in complete Freund's adjuvant and injected into five mice. Two additional injections of conjugated peptide in incomplete Freund's adjuvant occurred at three week intervals. Ten days after the final injection, serum from each of the five mice was tested for immunoreactivity with the twenty amino acid peptide by ELISA. In addition, the immunoreactivity of the sera were tested against intact 88C receptor expressed on the surface of 293 cells by fluorescence activated cell sorting (FACS). The mouse with the best anti-

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88C activity was chosen for spleen cell fusion and production of monoclonal antibodies by standard laboratory methods. Five monoclonal cell lines (227K, 227M, 227N, 227P, 227R) were established which produced antibodies that recognized the peptide by ELISA and the 88C protein on 293 cells by FACS.

5      Each antibody was shown to react only with 88C-expressing 293 cells, but not with 293 cells expressing the closely related MCP receptor (CCCKR-2). Each antibody was also shown to recognize 88C expressed transiently in COS cells.

Rabbit polyclonal antibodies were also generated against 88C. Two rabbits were injected with conjugated amino-terminal peptide as described above. The rabbits were further immunized by four additional injections of the conjugated amino-terminal peptide. Serum from each of the rabbits (2337J and 2470J) was tested by FACS of 293 cells expressing 88C. The sera specifically recognized 88C on the surface of 293 cells.

10     The five anti-88C monoclonal antibodies were tested for their ability to block infection of cells by SIV, the simian immunodeficiency virus closely related to HIV [Lehner, *et al.*, *Nature Medicine*, 2:767 (1996)]. Simian CD4<sup>+</sup> T cells, which are normally susceptible to infection by SIV, were incubated with the SIV<sub>mac</sub>32HJ5 clone in the presence of the anti-88C monoclonal antibody supernatants diluted 1:5. SIV infection was measured 20 by determining reverse transcriptase (RT) activity on day nine using the RT detection and quantification method (Quan-T-RT assay kit, Amersham, Arlington Heights, IL). Four of the antibodies were able to block SIV infection: antibody 227K blocked by 53%, 227M by 59%, 227N by 47% and 227P by 81%. Antibody 227R did not block SIV infection.

25     The five monoclonal antibodies raised against human 88C amino-terminal peptide were also tested for reactivity against macaque 88C (SEQ ID NO: X) (which has two amino acid differences from human 88C within the amino-terminal peptide region). The coding regions of human 88C and macaque 88C were cloned into the expression vector pcDNA3 (Invitrogen). These expression plasmids were used to transfect COS cells 30 using DEAE. The empty vector was used as a negative control. Three days after transfection, cells were harvested and incubated with the five anti-88C

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monoclonal antibodies and prepared for FACS. The results showed that four of the five antibodies (227K, 227M, 227N, 227P) recognized macaque 88C while one (227R) did not. All five antibodies recognized the transfected human 88C, and none cross-reacted with cells transfected with vector alone.

5

### Example 9

Additional methods may be used to identify ligands and modulators of the chemokine receptors of the invention.

In one embodiment, the invention comprehends a direct assay for ligands. Detectably labeled test compounds are exposed to membrane preparations presenting chemokine receptors in a functional conformation. For example, HEK-293 cells, or tissue culture cells, are transfected with an expression vehicle encoding a chemokine receptor. A membrane preparation is then made from the transfected cells expressing the chemokine receptor. The membrane preparation is exposed to <sup>125</sup>I-labeled test compounds (e.g., chemokines) and incubated under suitable conditions (e.g., 10 minutes at 37°C). The membranes, with any bound test compounds, are then collected on a filter by vacuum filtration and washed to remove unbound test compounds. The radioactivity associated with the bound test compound is then quantitated by subjecting the filters to liquid scintillation spectrophotometry. The specificity of test compound binding may be confirmed by repeating the assay in the presence of increasing quantities of unlabeled test compound and noting the level of competition for binding to the receptor. These binding assays can also identify modulators of chemokine receptor binding. The previously described binding assay may be performed with the following modifications. In addition to detectably labeled test compound, a potential modulator is exposed to the membrane preparation. An increased level of membrane-associated label indicates the potential modulator is an activator; a decreased level of membrane-associated label indicates the potential modulator is an inhibitor of chemokine receptor binding.

30

In another embodiment, the invention comprehends indirect assays for identifying receptor ligands that exploit the coupling of chemokine

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receptors to G proteins. As reviewed in *Linder et al., Sci. Am.*, 267:56-65 (1992), during signal transduction, an activated receptor interacts with a G protein, in turn activating the G protein. The G protein is activated by exchanging GDP for GTP. Subsequent hydrolysis of the G protein-bound GTP deactivates the G protein. One assay for G protein activity therefore monitors the release of  $^{32}\text{P}$ , from  $[\gamma-^{32}\text{P}]\text{-GTP}$ . For example, approximately 5  $\times 10^7$  HEK-293 cells harboring plasmids of the invention are grown in MEM + 10% FCS. The growth medium is supplemented with 5 mCi/ml [ $^{32}\text{P}$ ]-sodium phosphate for 2 hours to uniformly label nucleotide pools. The 10 cells are subsequently washed in a low-phosphate isotonic buffer. One aliquot of washed cells is then exposed to a test compound while a second aliquot of cells is treated similarly, but without exposure to the test compound. Following an incubation period (e.g., 10 minutes), cells are pelleted, lysed and nucleotide compounds fractionated using thin layer chromatography 15 developed with 1 M LiCl. Labeled GTP and GDP are identified by co-developing known standards. The labeled GTP and GDP are then quantitated by autoradiographic techniques that are standard in the art. Relatively high levels of  $^{32}\text{P}$ -labeled GDP identify test compounds as ligands. This type of GTP hydrolysis assay is also useful for the identification of modulators of chemokine receptor binding. The aforementioned assay is performed in the 20 presence of a potential modulator. An intensified signal resulting from a relative increase in GTP hydrolysis, producing  $^{32}\text{P}$ -labeled GDP, indicates a relative increase in receptor activity. The intensified signal therefore identifies the potential modulator as an activator. Conversely, a diminished relative 25 signal for  $^{32}\text{P}$ -labeled GDP, indicative of decreased receptor activity, identifies the potential modulator as an inhibitor of chemokine receptor binding.

The activities of G protein effector molecules (e.g., adenylyl cyclase, phospholipase C, ion channels, and phosphodiesterases) are also amenable to assay. Assays for the activities of these effector molecules have 30 been previously described. For example, adenylyl cyclase, which catalyzes the synthesis of cyclic adenosine monophosphate (cAMP), is activated by G proteins. Therefore, ligand binding to a chemokine receptor that activates a

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G protein, which in turn activates adenylyl cyclase, can be detected by monitoring cAMP levels in a recombinant host cell of the invention. Implementing appropriate controls understood in the art, an elevated level of intracellular cAMP can be attributed to a ligand-induced increase in receptor activity, thereby identifying a ligand. Again using controls understood in the art, a relative reduction in the concentration of cAMP would indirectly identify an inhibitor of receptor activity. The concentration of cAMP can be measured by a commercial enzyme immunoassay. For example, the BioTrak Kit provides reagents for a competitive immunoassay. (Amersham, Inc., Arlington Heights, IL). Using this kit according to the manufacturer's recommendations, a reaction is designed that involves competing unlabeled cAMP with cAMP conjugated to horseradish peroxidase. The unlabeled cAMP may be obtained, for example, from activated cells expressing the chemokine receptors of the invention. The two compounds compete for binding to an immobilized anti-cAMP antibody. After the competition reaction, the immobilized horseradish peroxidase-cAMP conjugate is quantitated by enzyme assay using a tetramethylbenzidine/H<sub>2</sub>O<sub>2</sub> single-pot substrate with detection of colored reaction products occurring at 450 nm. The results provide a basis for calculating the level of unlabeled cAMP, using techniques that are standard in the art. In addition to identifying ligands binding to chemokine receptors, the cAMP assay can also be used to identify modulators of chemokine receptor binding. Using recombinant host cells of the invention, the assay is performed as previously described, with the addition of a potential modulator of chemokine receptor activity. By using controls that are understood in the art, a relative increase or decrease in intracellular cAMP levels reflects the activation or inhibition of adenylyl cyclase activity. The level of adenylyl cyclase activity, in turn, reflects the relative activity of the chemokine receptor of interest. A relatively elevated level of chemokine receptor activity identifies an activator; a relatively reduced level of receptor activity identifies an inhibitor of chemokine receptor activity.

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While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those skilled in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(iii) NUMBER OF SEQUENCES: 20

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3383 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 55..1110

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(A) NAME/KEY: misc\_feature  
(D) OTHER INFORMATION: /= "88C polynucleotide and amino acid  
sequences"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGAAGAGCTG AGACATCCGT TCCCCTACAA GAAACTCTCC CGGGGTGGAA CAAG ATG  
Met

- 45 -

GAT TAT CAA GTG TCA AGT CCA ATC TAT GAC ATC AAT TAT TAT ACA TCG Asp Tyr Gln Val Ser Ser Pro Ile Tyr Asp Ile Asn Tyr Tyr Thr Ser 5 10 15	105
GAG CCC TGC CAA AAA ATC AAT GTG AAG CAA ATC GCA GCC CGC CTC CTG Glu Pro Cys Gln Lys Ile Asn Val Lys Gln Ile Ala Ala Arg Leu Leu 20 25 30	153
CCT CCG CTC TAC TCA CTG GTG TTC ATC TTT GGT TTT GTG GGC AAC ATG Pro Pro Leu Tyr Ser Leu Val Phe Ile Phe Gly Phe Val Gly Asn Met 35 40 45	201
CTG GTC ATC CTC ATC CTG ATA AAC TGC AAA AGG CTG AAG AGC ATG ACT Leu Val Ile Leu Ile Leu Ile Asn Cys Lys Arg Leu Lys Ser Met Thr 50 55 60 65	249
GAC ATC TAC CTG CTC AAC CTG GCC ATC TCT GAC CTG TTT TTC CTT CTT Asp Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Phe Phe Leu Leu 70 75 80	297
ACT GTC CCC TTC TGG GCT CAC TAT GCT GCC CAG TGG GAC TTT GGA Thr Val Pro Phe Trp Ala His Tyr Ala Ala Gln Trp Asp Phe Gly 85 90 95	345
AAT ACA ATG TGT CAA CTC TTG ACA GGG CTC TAT TTT ATA GGC TTC TTC Asn Thr Met Cys Gln Leu Leu Thr Gly Leu Tyr Phe Ile Gly Phe Phe 100 105 110	393
TCT GGA ATC TTC TTC ATC ATC CTC CTG ACA ATC GAT AGG TAC CTG GCT Ser Gly Ile Phe Phe Ile Ile Leu Leu Thr Ile Asp Arg Tyr Leu Ala 115 120 125	441
GTC GTC CAT GCT GTG TTT GCT TTA AAA GCC AGG ACG GTC ACC TTT GGG Val Val His Ala Val Phe Ala Leu Lys Ala Arg Thr Val Thr Phe Gly 130 135 140 145	489
GTG GTG ACA AGT GTG ATC ACT TGG GTG GTG GCT GTG TTT GCG TCT CTC Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala Ser Leu 150 155 160	537
CCA GGA ATC ATC TTT ACC AGA TCT CAA AAA GAA GGT CTT CAT TAC ACC Pro Gly Ile Ile Phe Thr Arg Ser Gln Lys Glu Gly Leu His Tyr Thr 165 170 175	585
TGC AGC TCT CAT TTT CCA TAC AGT CAG TAT CAA TTC TGG AAG AAT TTC Cys Ser Ser His Phe Pro Tyr Ser Gln Tyr Gln Phe Trp Lys Asn Phe 180 185 190	633
CAG ACA TTA AAG ATA GTC ATC TTG GGG CTG GTC CTG CCG CTG CTT GTC Gln Thr Leu Lys Ile Val Ile Leu Gly Leu Val Leu Pro Leu Leu Val 195 200 205	681
ATG GTC ATC TGC TAC TCG GGA ATC CTA AAA ACT CTG CTT CGG TGT CGA Met Val Ile Cys Tyr Ser Gly Ile Leu Lys Thr Leu Leu Arg Cys Arg 210 215 220 225	729
AAT GAG AAG AAG AGG CAC AGG GCT GTG AGG CTT ATC TTC ACC ATC ATG Asn Glu Lys Lys Arg His Arg Ala Val Arg Leu Ile Phe Thr Ile Met 230 235 240	777
ATT GTT TAT TTT CTC TTC TGG GCT CCC TAC AAC ATT GTC CTT CTC CTG Ile Val Tyr Phe Leu Phe Trp Ala Pro Tyr Asn Ile Val Leu Leu Leu 245 250 255	825
AAC ACC TTC CAG GAA TTC TTT GGC CTG AAT AAT TGC AGT AGC TCT AAC Asn Thr Phe Gln Glu Phe Phe Gly Leu Asn Asn Cys Ser Ser Ser Asn 260 265 270	873

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AGG TTG GAC CAA GCT ATG CAG GTG ACA GAG ACT CTT GGG ATG ACG CAC Arg Leu Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr His 275 280 285	921
TGC TGC ATC AAC CCC ATC ATC TAT GCC TTT GTC GGG GAG AAG TTC AGA Cys Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val Gly Glu Lys Phe Arg 290 295 300 305	969
AAC TAC CTC TTA GTC TTC CAA AAG CAC ATT GCC AAA CGC TTC TGC Asn Tyr Leu Leu Val Phe Phe Gln Lys His Ile Ala Lys Arg Phe Cys 310 315 320	1017
AAA TGC TGT TCT ATT TTC CAG CAA GAG GCT CCC GAG CGA GCA AGC TCA Lys Cys Cys Ser Ile Phe Gln Gln Glu Ala Pro Glu Arg Ala Ser Ser 325 330 335	1065
GTT TAC ACC CGA TCC ACT GGG GAG CAG GAA ATA TCT GTG GGC TTG Val Tyr Thr Arg Ser Thr Gly Glu Gln Glu Ile Ser Val Gly Leu 340 345 350	1110
TGACACGGAC TCAAGTGGGC TGGTGACCCA GTCAGAGTTG TGCACATGGC TTAGTTTCA 1170	
TACACAGCCT GGGCTGGGG TGCGGTGGGA GAGGTCTTT TTAAAAGGAA GTTACTGTTA 1230	
TAGAGGGTCT AAGATTCAATC CATTATTTG GCATCTGTT AAAGTAGATT AGATCTTTA 1290	
AGCCCATCAA TTATAGAAAG CCAAATCAAATATGTTGAT GAAAAATAGC AACCTTTTA 1350	
TCTCCCTTC ACATGCATCA AGTTATTGAC AAACTCTCCC TTCACTCCGA AAGTTCTTA 1410	
TGTATATTAA AAAGAAAGCC TCAGAGAATT GCTGATTCTT GAGTTAGTG ATCTGAACAG 1470	
AAATACCAAA ATTATTCAG AAATGTACAA CTTTTACCT AGTACAAGGC AACATATAGG 1530	
TTGTAAATGT GTTAAAACA GGTCTTGTC TTGCTATGGG GAGAAAAGAC ATGAATATGA 1590	
TTAGTAAAGA AATGACACTT TTCAATGTGTG ATTTCCCTC CAAGGTATGG TTAATAAGTT 1650	
TCACTGACTT AGAACCAAGGC GAGAGACTTG TGCGCTGGGA GAGCTGGGG AGCTTCTTAA 1710	
ATGAGAAGGA ATTTGAGTTG GATCATCTAT TGCTGGCAA GACAGAAGCC TCACTGCAAG 1770	
CACTGCATGG GCAAGCTTGG CTGTAGAAGG AGACAGAGCT GGTTGGGAAG ACATGGGGAG 1830	
GAAGGACAAG GCTAGATCAT GAAGAACCTT GACGGCATTG CTCCGTCTAA GTCATGAGCT 1890	
GAGCAGGGAG ATCCTGGTTG GTGTTGCAGA AGGTTACTC TGTGGCCAAA GGAGGGTCAG 1950	
GAAGGATGAG CATTAGGGC AAGGAGACCA CCAACAGCCC TCAGGTCAGG GTGAGGGATGG 2010	
CCTCTGCTAA GCTCAAGGCG TGAGGATGGG AAGGAGGGAG GTATTGCTAA GGATGGGAAG 2070	
GAGGGAGGTA TTCGTGCAGC ATATGAGGAT GCAGAGTCAG CAGAACTGGG GTGGATTTGG 2130	
TTTGGAAAGTG AGGGTCAGAG AGGAGTCAGA GAGAATCCCT AGTCTTCAAG CAGATTGGAG 2190	
AAACCTTGA AAAGACATCA AGCACAGAAG GAGGAGGGAG AGGTTAGGT CAAGAAGAAG 2250	
ATGGATTGGT GTAAAAGGAT GGGTCTGGTT TGCAGAGCTT GAACACAGTC TCACCCAGAC 2310	
TCCAGGCTGT CTTCACTGA ATGCTTCTGA CTTCATAGAT TTCCCTCCCA TCCCAGCTGA 2370	
AATACTGAGG GGTCTCCAGG AGGAGACTAG ATTTATGAAT ACACGAGGTA TGAGGTCTAG 2430	
GAACATACTT CAGCTCACAC ATGAGATCTA GGTGAGGATT GATTACCTAG TAGTCATTT 2490	
ATGGGTTGTT GGGAGGATTC TATGAGGCAA CCACAGGCAG CATTAGCAC ATACTACACA 2550	

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TTCAATAAGC ATCAAACCTCT TAGTTACTCA TTTCAGGGATA GCACTGAGCA AAGCATTGAG	2610
CAAAGGGGTC CCATATAGGT GAGGGAAAGCC TGAAAAACTA AGATGCTGCC TGCCCAGTGC	2670
ACACAAGTGT AGGTATCATT TTCTGCATT AACCGTCAAT AGGCAAAGGG GGGAAAGGGAC	2730
ATATTCACTT GGAAATAAGC TGCCTTGAGC CTTAAAACCC ACAAAAGTAC AATTTACCAG	2790
CCTCCGTATT TCAGACTGAA TGGGGGTGGG GGGGGCGCCT TAGGTACTTA TTCCAGATGC	2850
CTTCTCCAGA CAAACCAGAA GCAACAGAAA AAATCGTCTC TCCCTCCCTT TGAAATGAAT	2910
ATACCCCTTA GTGTTGGGT ATATTCACTT CAAAGGGAGA GAGAGAGGTT TTTTCTGTT	2970
CTTTCTCATA TGATTGTGCA CATACTTGAG ACTGTTTGAG ATTTGGGGGA TGGCTAAAAC	3030
CATCATAGTA CAGGTAAGGT GAGGGAAATAG TAAGTGGTGA GAACTACTCA GGGAAATGAAG	3090
GTGTCAGAAT AATAAGAGGT GCTACTGACT TTCTCAGCCT CTGAATATGA ACGGTGAGCA	3150
TTGTGGCTGT CAGCAGGAAG CAACGAAGGG AAATGTCTTT CCTTTTGCTC TTAAGTTGTG	3210
GAGAGTGCAGA CAGTAGGATA GGACCCCTACC CTCTGGGCCA AGTCAAAGAC ATTCTGACAT	3270
CTTAGTATT GCATATTCTT ATGTATGTGA AAGTTACAAA TTGCTTGAAA GAAAATATGC	3330
ATCTAATAAAA AAACACCTTC TAAAATAAAA AAAAAAAA AAAAAAAA AAA	3383

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 352 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (D) OTHER INFORMATION: /= "88C amino acid sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Tyr Gln Val Ser Ser Pro Ile Tyr Asp Ile Asn Tyr Tyr Thr			
1	5	10	15
Ser Glu Pro Cys Gln Lys Ile Asn Val Lys Gln Ile Ala Ala Arg Leu			
20	25	30	
Leu Pro Pro Leu Tyr Ser Leu Val Phe Ile Phe Gly Phe Val Gly Asn			
35	40	45	
Met Leu Val Ile Leu Ile Asn Cys Lys Arg Leu Lys Ser Met			
50	55	60	
Thr Asp Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Phe Phe Leu			
65	70	75	80
Leu Thr Val Pro Phe Trp Ala His Tyr Ala Ala Gln Trp Asp Phe			
85	90	95	
Gly Asn Thr Met Cys Gln Leu Leu Thr Gly Leu Tyr Phe Ile Gly Phe			
100	105	110	
Phe Ser Gly Ile Phe Phe Ile Ile Leu Leu Thr Ile Asp Arg Tyr Leu			
115	120	125	

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Ala Val Val His Ala Val Phe Ala Leu Lys Ala Arg Thr Val Thr Phe  
 130 135 140  
 Gly Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala Ser  
 145 150 155 160  
 Leu Pro Gly Ile Ile Phe Thr Arg Ser Gln Lys Glu Gly Leu His Tyr  
 165 170 175  
 Thr Cys Ser Ser His Phe Pro Tyr Ser Gln Tyr Gln Phe Trp Lys Asn  
 180 185 190  
 Phe Gln Thr Leu Lys Ile Val Ile Leu Gly Leu Val Leu Pro Leu Leu  
 195 200 205  
 Val Met Val Ile Cys Tyr Ser Gly Ile Leu Lys Thr Leu Leu Arg Cys  
 210 215 220  
 Arg Asn Glu Lys Lys Arg His Arg Ala Val Arg Leu Ile Phe Thr Ile  
 225 230 235 240  
 Met Ile Val Tyr Phe Leu Phe Trp Ala Pro Tyr Asn Ile Val Leu Leu  
 245 250 255  
 Leu Asn Thr Phe Gln Glu Phe Phe Gly Leu Asn Asn Cys Ser Ser Ser  
 260 265 270  
 Asn Arg Leu Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr  
 275 280 285  
 His Cys Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val Gly Glu Lys Phe  
 290 295 300  
 Arg Asn Tyr Leu Leu Val Phe Phe Gln Lys His Ile Ala Lys Arg Phe  
 305 310 315 320  
 Cys Lys Cys Cys Ser Ile Phe Gln Gln Glu Ala Pro Glu Arg Ala Ser  
 325 330 335  
 Ser Val Tyr Thr Arg Ser Thr Gly Glu Gln Glu Ile Ser Val Gly Leu  
 340 345 350

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1915 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 362..1426

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88-2B polynucleotide and amino acid sequences"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATAATAATGA TTATTATATT GTTATCATTA TCTAGCCTGT TTTTCCTGT TTTGTATTTC	60
TTCCCTTAAA TGCTTTCAGA AATCTGTATC CCCATTCTTC ACCACCACCC CACAACATT	120

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CTGCTTCTTT TCCCCATGCCG GGTCAATGCTA ACTTTGAAAG CTTCAAGCTCT TTCCCTTCCTC	180
AATCCTTTTC CTGGCACCTC TGATATGCCT TTTGAAATTG ATGTTAAAGA ATCCCCTAGGC	240
TGCTATCACAC TGTGGCATCT TTGTTGAGTA CATGAATAAA TCAACTGGTG TGTTTACGA	300
AGGATGATTA TGCTTCATTG TGGGATTGTA TTTTCTTCT TCTATCACAG GGAGAAGTGA	360
A ATG ACA ACC TCA CTA GAT ACA GTT GAG ACC TTT GGT ACC ACA TCC Met Thr Thr Ser Leu Asp Thr Val Glu Thr Phe Gly Thr Thr Ser	406
1 5 10 15	
TAC TAT GAT GAC GTG GGC CTG CTC TGT GAA AAA GCT GAT ACC AGA GCA Tyr Tyr Asp Asp Val Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala	454
20 25 30	
CTG ATG GCC CAG TTT GTG CCC CCG CTG TAC TCC CTG GTG TTC ACT GTG Leu Met Ala Gln Phe Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Val	502
35 40 45	
GGC CTC TTG GGC AAT GTG GTG GTG ATG ATC CTC ATA AAA TAC AGG Gly Leu Leu Gly Asn Val Val Val Met Ile Leu Ile Lys Tyr Arg	550
50 55 60	
AGG CTC CGA ATT ATG ACC AAC ATC TAC CTG CTC AAC CTG GCC ATT TCG Arg Leu Arg Ile Met Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser	598
65 70 75	
GAC CTG CTC TTC CTC GTC ACC CTT CCA TTC TGG ATC CAC TAT GTC AGG Asp Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg	646
80 85 90 95	
GGG CAT AAC TGG GTT TTT GGC CAT GGC ATG TGT AAG CTC CTC TCA GGG Gly His Asn Trp Val Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly	694
100 105 110	
TTT TAT CAC ACA GGC TTG TAC AGC GAG ATC TTT TTC ATA ATC CTG CTG Phe Tyr His Thr Gly Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu	742
115 120 125	
ACA ATC GAC AGG TAC CTG GCC ATT GTC CAT GCT GTG TTT GCC CTT CGA Thr Ile Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu Arg	790
130 135 140	
GCC CGG ACT GTC ACT TTT GGT GTC ATC ACC AGC ATC GTC ACC TGG GGC Ala Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly	838
145 150 155	
CTG GCA GTG CTA GCA GCT CTT CCT GAA TTT ATC TTC TAT GAG ACT GAA Leu Ala Val Leu Ala Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu	886
160 165 170 175	
GAG TTG TTT GAA GAG ACT CTT TGC AGT GCT CTT TAC CCA GAG GAT ACA Glu Leu Phe Glu Glu Thr Leu Cys Ser Ala Leu Tyr Pro Glu Asp Thr	934
180 185 190	
GTA TAT AGC TGG AGG CAT TTC CAC ACT CTG AGA ATG ACC ATC TTC TGT Val Tyr Ser Trp Arg His Phe His Thr Leu Arg Met Thr Ile Phe Cys	982
195 200 205	
CTC GTT CTC CCT CTG CTC GTT ATG GCC ATC TGC TAC ACA GGA ATC ATC Leu Val Leu Pro Leu Leu Val Met Ala Ile Cys Tyr Thr Gly Ile Ile	1030
210 215 220	
AAA ACG CTG CTG AGG TGC CCC AGT AAA AAA AAG TAC AAG GCC ATC CGG Lys Thr Leu Leu Arg Cys Pro Ser Lys Lys Lys Tyr Lys Ala Ile Arg	1078
225 230 235	

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CTC ATT TTT GTC ATC ATG GCG GTG TTT TTC ATT TTC TGG ACA CCC TAC Leu Ile Phe Val Ile Met Ala Val Phe Phe Ile Phe Trp Thr Pro Tyr 240 245 250 255	1126
AAT GTG GCT ATC CTT CTC TCT TCC TAT CAA TCC ATC TTA TTT GGA AAT Asn Val Ala Ile Leu Leu Ser Ser Tyr Gln Ser Ile Leu Phe Gly Asn 260 265 270	1174
GAC TGT GAG CGG AGC AAG CAT CTG GAC CTG GTC ATG CTG GTG ACA GAG Asp Cys Glu Arg Ser Lys His Leu Asp Leu Val Met Leu Val Thr Glu 275 280 285	1222
GTG ATC GCC TAC TCC CAC TGC TGC ATG AAC CCG GTG ATC TAC GCC TTT Val Ile Ala Tyr Ser His Cys Cys Met Asn Pro Val Ile Tyr Ala Phe 290 295 300	1270
GTT GGA GAG AGG TTC CGG AAG TAC CTG CGC CAC TTC TTC CAC AGG CAC Val Gly Glu Arg Phe Arg Lys Tyr Leu Arg His Phe Phe His Arg His 305 310 315	1318
TTG CTC ATG CAC CTG GGC AGA TAC ATC CCA TTC CTT CCT AGT GAG AAG Leu Leu Met His Leu Gly Arg Tyr Ile Pro Phe Leu Pro Ser Glu Lys 320 325 330 335	1366
CTG GAA AGA ACC AGC TCT GTC TCT CCA TCC ACA GCA GAG CCG GAA CTC Leu Glu Arg Thr Ser Ser Val Ser Pro Ser Thr Ala Glu Pro Glu Leu 340 345 350	1414
TCT ATT GTG TTT TAGTCAGAT GCAGAAAATT GCCTAAAGAG GAAGGACCAA Ser Ile Val Phe 355	1466
GGAGATGAAG CAAACACATT AAGCCTTCCA CACTCACCTC TAAAACAGTC CTTCAAACCTT CCAGTGCAAC ACTGAAGCTC TTGAAGACAC TGAAATATAAC ACACAGCAGT AGCAGTAGAT	1526
GCATGTACCC TAAGGTCAATT ACCACAGGCC AGGGGCTGGG CAGCGTACTC ATCATCAACC CTAAAAAGCA GAGCTTGCT TCTCTCTCTA AAATGAGTTA CCTACATTTT AATGCACCTG	1586
AATGTTAGAT AGTTACTATA TGCCGCTACA AAAAGGTAAA ACTTTTATA TTTTATACAT TAACTTCAGC CAGCTATTGA TATAAATAAA ACATTTCAC ACAATACAAT AAGTTAACTA	1646
TTTTATTTTC TAATGTGCCT AGTTCTTCC CTGCTTAATG AAAAGCTTGT TTTTCAGTG TGAATAAATA ATCGTAAGCA ACAAAAAAA	1706
(2) INFORMATION FOR SEQ ID NO:4:	1766
(i) SEQUENCE CHARACTERISTICS:	1826
(A) LENGTH: 355 amino acids	1886
(B) TYPE: amino acid	1915
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: protein	
(ix) FEATURE:	
(A) NAME/KEY: misc_feature	
(D) OTHER INFORMATION: /= "88-2B amino acid sequence"	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
Met Thr Thr Ser Leu Asp Thr Val Glu Thr Phe Gly Thr Thr Ser Tyr 1 5 10 15	

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Tyr Asp Asp Val Gly Leu Leu Cys Glu Lys Ala Asp Thr Arg Ala Leu  
20 25 30

Met Ala Gln Phe Val Pro Pro Leu Tyr Ser Leu Val Phe Thr Val Gly  
35 40 45

Leu Leu Gly Asn Val Val Val Met Ile Leu Ile Lys Tyr Arg Arg  
50 55 60

Leu Arg Ile Met Thr Asn Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp  
65 70 75 80

Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg Gly  
85 90 95

His Asn Trp Val Phe Gly His Gly Met Cys Lys Leu Leu Ser Gly Phe  
100 105 110

Tyr His Thr Gly Leu Tyr Ser Glu Ile Phe Phe Ile Leu Leu Thr  
115 120 125

Ile Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu Arg Ala  
130 135 140

Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly Leu  
145 150 155 160

Ala Val Leu Ala Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu Glu  
165 170 175

Leu Phe Glu Glu Thr Leu Cys Ser Ala Leu Tyr Pro Glu Asp Thr Val  
180 185 190

Tyr Ser Trp Arg His Phe His Thr Leu Arg Met Thr Ile Phe Cys Leu  
195 200 205

Val Leu Pro Leu Leu Val Met Ala Ile Cys Tyr Thr Gly Ile Ile Lys  
210 215 220

Thr Leu Leu Arg Cys Pro Ser Lys Lys Tyr Lys Ala Ile Arg Leu  
225 230 235 240

Ile Phe Val Ile Met Ala Val Phe Phe Ile Phe Trp Thr Pro Tyr Asn  
245 250 255

Val Ala Ile Leu Leu Ser Ser Tyr Gln Ser Ile Leu Phe Gly Asn Asp  
260 265 270

Cys Glu Arg Ser Lys His Leu Asp Leu Val Met Leu Val Thr Glu Val  
275 280 285

Ile Ala Tyr Ser His Cys Cys Met Asn Pro Val Ile Tyr Ala Phe Val  
290 295 300

Gly Glu Arg Phe Arg Lys Tyr Leu Arg His Phe Phe His Arg His Leu  
305 310 315 320

Leu Met His Leu Gly Arg Tyr Ile Pro Phe Leu Pro Ser Glu Lys Leu  
325 330 335

Glu Arg Thr Ser Ser Val Ser Pro Ser Thr Ala Glu Pro Glu Leu Ser  
340 345 350

Ile Val Phe  
355

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## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "V28degf2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACGGATCCA TYGAYAGRATA CCTGGCYATY GTCC

34

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "V28degr2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTAAAGCTTT TRTAGGGDGT CCAYAAGAGY AA

32

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88c-r4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATAAGCCTC ACAGCCCTGT G

21

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88c-rlb"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCTAAGCTTG ATGACTATCT TTAATGTC

28

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88-2B-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCTCTAGAC TAAAACACAA TAGAGAG

27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88-2B-5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCTAAGCTTA TCACAGGGAG AAGTGAAATG

30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88-2B-f1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGTGCTAGCA GCTCTTCCTG

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88-2B-r1"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGCAGCGTT TTGATGATT<sup>C</sup>

20

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88C-f1"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTGTTTGCT TTAAAAGCC

19

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "88C-r3"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TAAGCCTCAC AGCCCTG

17

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "CCCKR1(2)-5■ Primer"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGTAAGCTTA GAGAAGCCGG GATGGGAA

28

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: YES

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (D) OTHER INFORMATION: /= "CCCKR-3■ Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCTCTAGAG TCAGAGACCA GCAGA

25

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GACAAGCTTC ACAGGGTGGA ACAAGATG

28

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCTCTAGAC CACTTGAGTC CGTGTCA

27

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1059 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1056

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

- 56 -

ATG GAC TAT CAA GTG TCA AGT CCA ACC TAT GAC ATC GAT TAT TAT ACA Met Asp Tyr Gln Val Ser Ser Pro Thr Tyr Asp Ile Asp Tyr Tyr Thr 1 5 10 15	48
TCG GAA CCC TGC CAA AAA ATC AAT GTG AAA CAA ATC GCA GCC CGC CTC Ser Glu Pro Cys Gln Lys Ile Asn Val Lys Gln Ile Ala Ala Arg Leu 20 25 30	96
CTG CCT CCG CTC TAC TCA CTG GTG TTC ATC TTT GGT TTT GTG GGC AAC Leu Pro Pro Leu Tyr Ser Leu Val Phe Ile Phe Gly Phe Val Gly Asn 35 40 45	144
ATA CTG GTC GTC CTC ATC CTG ATA AAC TGC AAA AGG CTG AAA AGC ATG Ile Leu Val Val Leu Ile Leu Ile Asn Cys Lys Arg Leu Lys Ser Met 50 55 60	192
ACT GAC ATC TAC CTG CTC AAC CTG GCC ATC TCT GAC CTG CTT TTC CTT Thr Asp Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Leu Phe Leu 65 70 75 80	240
CTT ACT GTC CCC TTC TGG GCT CAC TAT GCT GCT GCC CAG TGG GAC TTT Leu Thr Val Pro Phe Trp Ala His Tyr Ala Ala Gln Trp Asp Phe 85 90 95	288
GGA AAT ACA ATG TGT CAA CTC TTG ACA GGG CTC TAT TTT ATA GGC TTC Gly Asn Thr Met Cys Gln Leu Leu Thr Gly Leu Tyr Phe Ile Gly Phe 100 105 110	336
TTC TCT GGA ATC TTC ATC ATC CTC CTG ACA ATC GAT AGG TAC CTG Phe Ser Gly Ile Phe Phe Ile Ile Leu Leu Thr Ile Asp Arg Tyr Leu 115 120 125	384
GCT ATC GTC CAT GCT GTG TTT GCT TTA AAA GCC AGG ACA GTC ACC TTT Ala Ile Val His Ala Val Phe Ala Leu Lys Ala Arg Thr Val Thr Phe 130 135 140	432
GGG GTG GTG ACA AGT GTG ATC ACT TGG GTG GTG GCT GTG TTT GCC TCT Gly Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala Ser 145 150 155 160	480
CTC CCA GGA ATC ATC TTT ACC AGA TCT CAG AGA GAA GGT CTT CAT TAC Leu Pro Gly Ile Ile Phe Thr Arg Ser Gln Arg Glu Gly Leu His Tyr 165 170 175	528
ACC TGC AGC TCT CAT TTT CCA TAC AGT CAG TAT CAA TTC TGG AAG AAT Thr Cys Ser Ser His Phe Pro Tyr Ser Gln Tyr Gln Phe Trp Lys Asn 180 185 190	576
TTT CAG ACA TTA AAG ATG GTC ATC TTG GGG CTG GTC CTG CCG CTG CTT Phe Gln Thr Leu Lys Met Val Ile Leu Gly Leu Val Leu Pro Leu Leu 195 200 205	624
GTC ATG GTC ATC TGC TAC TCG GGA ATC CTG AAA ACT CTG CTT CGG TGT Val Met Val Ile Cys Tyr Ser Gly Ile Leu Lys Thr Leu Leu Arg Cys 210 215 220	672
CGA AAC GAG AAG AAG AGG CAC AGG GCT GTG AGG CTT ATC TTC ACC ATC Arg Asn Glu Lys Lys Arg His Arg Ala Val Arg Leu Ile Phe Thr Ile 225 230 235 240	720
ATG ATT GTT TAT TTT CTC TTG TGG GCT CCC TAC AAC ATT GTC CTT CTC Met Ile Val Tyr Phe Leu Leu Trp Ala Pro Tyr Asn Ile Val Leu Leu 245 250 255	768
CTG AAC ACC TTC CAG GAA TTC TTT GGC CTG AAT AAT TGC AGT AGC TCT Leu Asn Thr Phe Gln Glu Phe Phe Gly Leu Asn Asn Cys Ser Ser Ser 260 265 270	816

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AAC AGG TTG GAC CAA GCC ATG CAG GTG ACA GAG ACT CTT GGG ATG ACA	864		
Asn Arg Leu Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr			
275	280	285	
CAC TGC TGC ATC AAC CCC ATC ATC TAT GCC TTT GTC GGG GAG AAG TTC	912		
His Cys Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val Gly Glu Lys Phe			
290	295	300	
AGA AAC TAC CTC TTA GTC TTC TTC CAA AAG CAC ATT GCC AAA CGC TTC	960		
Arg Asn Tyr Leu Leu Val Phe Phe Gln Lys His Ile Ala Lys Arg Phe			
305	310	315	320
TGC AAA TGC TGT TCC ATT TTC CAG CAA GAG GCT CCC GAG CGA GCA AGT	1008		
Cys Lys Cys Cys Ser Ile Phe Gln Gln Glu Ala Pro Glu Arg Ala Ser			
325	330	335	
TCA GTT TAC ACC CGA TCC ACT GGG GAG CAG GAA ATA TCT GTG GGC TTG	1056		
Ser Val Tyr Thr Arg Ser Thr Gly Glu Gln Glu Ile Ser Val Gly Leu			
340	345	350	
TGA	1059		

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 352 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Met	Asp	Tyr	Gln	Val	Ser	Ser	Pro	Thr	Tyr	Asp	Ile	Asp	Tyr	Tyr	Thr
1				5					10				15		
Ser	Glu	Pro	Cys	Gln	Lys	Ile	Asn	Val	Lys	Gln	Ile	Ala	Ala	Arg	Leu
					20				25				30		
Leu	Pro	Pro	Leu	Tyr	Ser	Leu	Val	Phe	Ile	Phe	Gly	Phe	Val	Gly	Asn
							35		40			45			
Ile	Leu	Val	Val	Leu	Ile	Leu	Ile	Asn	Cys	Lys	Arg	Leu	Lys	Ser	Met
							50		55			60			
Thr	Asp	Ile	Tyr	Leu	Leu	Asn	Leu	Ala	Ile	Ser	Asp	Leu	Leu	Phe	Leu
							65		70			75			80
Leu	Thr	Val	Pro	Phe	Trp	Ala	His	Tyr	Ala	Ala	Ala	Gln	Trp	Asp	Phe
							85				90			95	
Gly	Asn	Thr	Met	Cys	Gln	Leu	Leu	Thr	Gly	Leu	Tyr	Phe	Ile	Gly	Phe
							100		105			110			
Phe	Ser	Gly	Ile	Phe	Phe	Ile	Ile	Leu	Leu	Thr	Ile	Asp	Arg	Tyr	Leu
							115		120			125			
Ala	Ile	Val	His	Ala	Val	Phe	Ala	Leu	Lys	Ala	Arg	Thr	Val	Thr	Phe
							130		135			140			
Gly	Val	Val	Thr	Ser	Val	Ile	Thr	Trp	Val	Val	Ala	Val	Phe	Ala	Ser
							145		150			155			160
Leu	Pro	Gly	Ile	Ile	Phe	Thr	Arg	Ser	Gln	Arg	Glu	Gly	Leu	His	Tyr
							165				170			175	

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Thr Cys Ser Ser His Phe Pro Tyr Ser Gln Tyr Gln Phe Trp Lys Asn  
180 185 190

Phe Gln Thr Leu Lys Met Val Ile Leu Gly Leu Val Leu Pro Leu Leu  
195 200 205

Val Met Val Ile Cys Tyr Ser Gly Ile Leu Lys Thr Leu Leu Arg Cys  
210 215 220

Arg Asn Glu Lys Lys Arg His Arg Ala Val Arg Leu Ile Phe Thr Ile  
225 230 235 240

Met Ile Val Tyr Phe Leu Leu Trp Ala Pro Tyr Asn Ile Val Leu Leu  
245 250 255

Leu Asn Thr Phe Gln Glu Phe Phe Gly Leu Asn Asn Cys Ser Ser Ser  
260 265 270

Asn Arg Leu Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr  
275 280 285

His Cys Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val Gly Glu Lys Phe  
290 295 300

Arg Asn Tyr Leu Leu Val Phe Phe Gln Lys His Ile Ala Lys Arg Phe  
305 310 315 320

Cys Lys Cys Cys Ser Ile Phe Gln Gln Glu Ala Pro Glu Arg Ala Ser  
325 330 335

Ser Val Tyr Thr Arg Ser Thr Gly Glu Gln Glu Ile Ser Val Gly Leu  
340 345 350

CLAIMS

We claim:

1. A purified and isolated polynucleotide encoding the amino acid sequence of chemokine receptor 88-2B set out in SEQ ID NO:4.
2. A polynucleotide according to claim 1 wherein the polynucleotide is DNA.
3. A polynucleotide according to claim 2 wherein the polynucleotide is genomic DNA.
4. A polynucleotide according to claim 2 wherein the polynucleotide is cDNA.
5. A polynucleotide according to claim 1 which is a wholly or partially chemically synthesized DNA.
6. An RNA transcript of the polynucleotide of claim 2.
7. A cDNA according to claim 4 comprising the DNA of SEQ ID NO:3.
8. A biologically functional DNA vector comprising a DNA according to claim 2.
9. A vector according to claim 8 wherein said DNA is operatively linked to a DNA expression control sequence.
10. A host cell stably transformed or transfected with a DNA according to claim 1 in a manner allowing expression of said DNA.

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11. A method for producing an 88-2B polypeptide comprising the steps of growing a host cell according to claim 10 in a suitable nutrient medium and isolating said polypeptide from said cell or medium.

12. A polynucleotide encoding an 88-2B polypeptide wherein said polynucleotide hybridizes under stringent hybridization conditions to the polynucleotide of SEQ ID NO: 3.

13. A purified and isolated polypeptide comprising the chemokine receptor 88-2B amino acid sequence set out in SEQ ID NO:4.

14. An antibody product that specifically binds a polypeptide comprising the 88-2B amino acid sequence set out in SEQ ID NO:4.

15. A hybridoma producing an antibody product according to claim 14.

16. A purified and isolated polynucleotide encoding the amino acid sequence of chemokine receptor 88C set out in SEQ ID NO:2.

17. A polynucleotide according to claim 16 wherein the polynucleotide is DNA.

18. A polynucleotide according to claim 17 wherein the polynucleotide is genomic DNA.

19. A polynucleotide according to claim 17 wherein the polynucleotide is a cDNA.

20. A polynucleotide according to claim 16 which is a wholly or partially chemically synthesized DNA.

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21. An RNA transcript of the polynucleotide of claim 17.
22. A cDNA according to claim 19 comprising the DNA of SEQ ID NO:1.
23. A biologically functional DNA vector comprising a DNA according to claim 17.
24. A vector according to claim 23 wherein said DNA is operatively linked to a DNA expression control sequence.
25. A host cell stably transformed or transfected with a DNA according to claim 16 in a manner allowing expression of said DNA.
26. A method for producing an 88C polypeptide comprising the steps of growing a host cell according to claim 25 in a suitable nutrient medium and isolating said polypeptide from said cell or medium.
27. A polynucleotide encoding an 88C polypeptide wherein said polynucleotide hybridizes under stringent hybridization conditions to the polynucleotide of SEQ ID NO: 1.
28. A purified and isolated polypeptide comprising the chemokine receptor 88C amino acid sequence set out in SEQ ID NO:2.
29. An antibody product that specifically binds a polypeptide comprising the 88C amino acid sequence set out in SEQ ID NO:2.
30. A hybridoma producing an antibody product according to claim 29.
31. Hybridoma cell line 227K.

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32. Hybridoma cell line 227M.
33. Hybridoma cell line 227N.
34. Hybridoma cell line 227P.
35. Hybridoma cell line 227R.
36. A purified and isolated polynucleotide encoding the amino acid sequence of macaque chemokine receptor 88C set out in SEQ ID NO: 20.
37. A polynucleotide according to claim 36 wherein the polynucleotide is DNA.
38. A polynucleotide according to claim 37 wherein the polynucleotide is genomic DNA.
39. A polynucleotide according to claim 37 wherein the polynucleotide is a cDNA.
40. A polynucleotide according to claim 36 which is a wholly or partially chemically synthesized DNA.
41. An RNA transcript of the polynucleotide of claim 37.
42. A cDNA according to claim 39 comprising the DNA of SEQ ID NO:1.
43. A biologically functional DNA vector comprising a DNA according to claim 37.

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44. A vector according to claim 43 wherein said DNA is operatively linked to a DNA expression control sequence.

45. A host cell stably transformed or transfected with a DNA according to claim 36 in a manner allowing expression of said DNA.

46. A method for producing a macaque 88C polypeptide comprising the steps of growing a host cell according to claim 45 in a suitable nutrient medium and isolating said polypeptide from said cell or medium.

47. A polynucleotide encoding an 88C polypeptide wherein said polynucleotide hybridizes under stringent hybridization conditions to the polynucleotide of SEQ ID NO: 19.

48. A purified and isolated polypeptide comprising the macaque chemokine receptor 88C amino acid sequence set out in SEQ ID NO:20.

49. An antibody product that specifically binds a polypeptide comprising the 88C amino acid sequence set out in SEQ ID NO:20.

50. A hybridoma producing an antibody product according to claim 49.



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/12, C07K 14/705, 16/28, C12N 5/20</b>		A3	(11) International Publication Number: <b>WO 97/22698</b>
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(22) International Filing Date: <b>20 December 1996 (20.12.96)</b>		(81) Designated States: AU, BR, CA, CN, CZ, FI, HU, JP, MX, NO, PL, RU, SK, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: <b>08/575,967 20 December 1995 (20.12.95) US 08/661,393 7 June 1996 (07.06.96) US</b>		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: ICOS CORPORATION [US/US]; 22021 20th Avenue S.E., Bothell, WA 98021 (US).		(88) Date of publication of the international search report: <b>12 September 1997 (12.09.97)</b>	
(72) Inventors: GRAY, Patrick, W.; 1600 40th Avenue, Seattle, WA 98122 (US). SCHWEICKART, Vicki, L.; 1421 Orange Place North, Seattle, WA 98109 (US). RAPORT, Carol, J.; 2300 211th Street, S.E., Bothell, WA 98021 (US).			
(74) Agent: BORUN, Michael, F.; Marshall, O'Toole, Gerstein, Murray & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606-6402 (US).			

(54) Title: CHEMOKINE RECEPTORS 88-2B[CKR-3] AND 88C AND THEIR ANTIBODIES

(57) Abstract

The present invention provides polynucleotides that encode the chemokine receptors 88-2B or 88C and materials and methods for the recombinant production of these two chemokine receptors. Also provided are assays utilizing the polynucleotides which facilitate the identification of ligands and modulators of the chemokine receptors. Receptor fragments, ligands, modulators, and antibodies are useful in the detection and treatment of disease states associated with the chemokine receptors such as atherosclerosis, rheumatoid arthritis, tumor growth suppression, asthma, viral infection, AIDS, and other inflammatory conditions.

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/20759

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 C12N15/12 C07K14/705 C07K16/28 C12N5/20

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, 14 July 1995, MD US, pages 16491-16494, XP002029908 COMBADIERE, C. ET AL. : "Cloning and functional expression of a human eosinophil CC Chemokine Receptor" see the whole document ---	1-15
P,X	WO 96 22371 A (LEUKOSITE INC ; BRIGHAM & WOMENS HOSPITAL (US); CHILDRENS MEDICAL C) 25 July 1996 see claims 1-29, 38, 39 ---	1-15
P,X	WO 96 39437 A (HUMAN GENOME SCIENCES INC ; LI YI (US); RUBEN STEVEN M (US)) 12 December 1996 see page 33, line 7 - page 34, line 11; claim 1 ---	16-50

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

15 July 1997

Date of mailing of the international search report

22.07.97

Name and mailing address of the ISA

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Authorized officer

Nauche, S

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/20759

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BIOCHEMISTRY, MAR 19 1996, 35 (11) P3362-7, UNITED STATES, XP002035224 SAMSON M ET AL: "Molecular cloning and functional expression of a new human CC-chemokine receptor gene." see the whole document ---	16-50
P,X	NATURE, JUN 20 1996, 381 (6584) P667-73, ENGLAND, XP002035225 DRAGIC T ET AL: "HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5 [see comments]" see the whole document ---	16-50
P,X	NATURE, JUN 20 1996, 381 (6584) P661-6, ENGLAND, XP002035226 DENG H ET AL: "Identification of a major co-receptor for primary isolates of HIV-1 [see comments]" see the whole document -----	16-50

# **INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 96/20759

**Box 1** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:



**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheets

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

## **Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 20759

FURTHER INFORMATION CONTINUED FROM PCT/USA/210

1. Claims: 1-15 A chemokine receptor 88-2B [ CKR-3 chemokine receptor], sequence encoding said receptor. Expression vector and recombinant host cells for the production of chemokine receptor 88-2B [ CKR-3 chemokine receptor]. Antibodies immunoreactive with chemokine receptor 88-2B [ CKR-3 chemokine receptor]. Hybridoma producing said antibodies.
2. Claims: 16-50 chemokine receptor 88-C, sequence encoding said receptor. Expression vector and recombinant host cells for the production of chemokine receptor 88-C. Antibodies immunoreactive with chemokine receptor 88-C. Hybridoma producing said antibodies

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/20759

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9622371 A	25-07-96	AU 5020296 A	07-08-96
WO 9639437 A	12-12-96	AU 2663295 A	24-12-96

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